FINAL REPORT

Development and Application of a Physiological-based Framework for Assessing the Biological Significance of Military Activities on Threatened and Endangered Animal Species

SERDP Project SI-1395

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14. ABSTRACT

This main objective of this SERDP project was to develop and apply an integrated multivariate bioindicator approach for assessing effects of military activities and other environmental factors on TER-S and to serve as an operational framework for resource managers at military sites to evaluate the fitness of TER-S. Both level of military activity and habitat quality were influential factors in dictating the magnitude and nature of health responses in gopher tortoises at Camp Shelby, MS. with habitat apparently having the greater (relative) effect. The fitness of tortoise populations is primarily related to habitat quality because habitat quality influences population size and population size is directly related to genetic fitness and therefore to overall health of tortoises. A multivariate discriminant analysis revealed that a reduced set (7-8) of bioindicators from a total set of about 40 measured variables effectively predicted tortoise health. Using multiple response variables at different levels of biological organization is necessary in helping to understand causal relationships between environmental factors, organism response, and the biological relevance of such responses. Results provide by this study suggest that some effective environmental management options are available for mitigating or minimizing any effects due to military activity on sensitive TER-S such as the gopher tortoise. Identification of those specific actions and environmental variables responsible for injury to TER-S should reduce the uncertainty of environmental management and regulatory decisions resulting in an increased ability to predict the consequences of specific actions or activities on military ranges.

15. SUBJECT TERMS

multivariate bioindicators, gopher tortoise, habitat quality, military activity, population/genetic fitness, Camp Shelby, environmental management

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LIST OF ACRONYMS

ACTH	Adrenocorticotropic hormone
AFB	Air Force Base
ANOVA	Analysis of variance
AST	Aminotransferase
BCI	Body condition index
DoD	Department of Defense
GIS	Geographic Information System
HWE	Hardy Weinberg equilibrium
IBD	Isolation-by-distance model
LDH	Lactate dehydrogenase
PCR	Polymerase chain reaction
TER-S	Threatened, endangered, and at-risk species
URTD	Upper respiratory tract disease

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EXECUTIVE SUMMARY

The primary objective of this project was to develop and apply a bioassessment approach that can be used by environmental resource managers at military installations to evaluate the fitness of Threatened, Endangered, and at-Risk Species (TER-S) through the measurement of a selected suite of sensitive and rapidly-responding bioindicators. This study applied an empirical, proof-of-concept approach to evaluate if these sensitive and rapidly-responding bioindicators could be used by Department of Defense (DoD) resource managers as a practical and cost-effective tool for evaluating the health status of TER-S.

This SERDP project has as its main goal the development and application of an integrated and multivariate bioindicator (physiological) approach for assessing effects of military activities and other environmental factors such as habitat on the health and fitness of a representative TER-S at Camp Shelby and at Eglin AFB. Due primarily to the exceptional drought in the Southeastern US over the entire period of this project during which frogs could not be collected at Eglin AFB, the bioindicator studies originally planned for gopher frogs (*Rana caprio*) had to be discontinued after the habitat and chemical characterization phase of the study. Consequently, the remaining SERDP funds originally earmarked for this effort (sampling of frogs, processing and analysis of samples, etc) were redirected toward extension of some additional gopher tortoise studies including diet and food analysis, additional reproductive fitness studies, and landscape population genetic studies.

Because wildlife resources on military ranges have the potential to be affected by a variety of environmental factors or stressors, the experimental field design of this project included level of military activity and habitat quality as the main treatment variables. Inclusion of multiple treatment effects into the field design helps to determine the relative contribution of each factor on the health or fitness of the TER-S of concern. For our studies at Camp Shelby, both level of military activity and habitat quality were influential factors in dictating the magnitude and nature of health responses in the gopher tortoise (*Gopherus polyphemus*) with habitat apparently having the greater (relative) effect.

To assess the health or fitness of tortoises residing in areas characterized by different levels of military activity and habitat quality, we applied a multivariate bioindicator approach that incorporated all of the measured bioindicators in an integrated analysis. Using all the measured bioindicators in the integrated discriminant analysis revealed that both habitat quality and level of military activity are important in influencing the health and condition of tortoises at Camp Shelby. A reduced set (7-8) of bioindicators from a total set of about 40 measured variables effectively predicted tortoise health based on differences among the experimental treatments. As functional response groups, the organ dysfunction, carbohydrate-protein metabolism, and stress hormones are the key diagnostic responses as a group that are indicative of habitat effects while bioenergetic, electrolyte homeostasis, and oxidative stress indicators as a group are the primary diagnostic responses that are the key indicators of military activity effects on tortoises.

The fact that a number and variety of physiological response groups are important in discriminating among experimental treatments illustrates the importance of using multiple response indicators (or functional response groups) to assess the effects of environmental factors (stressors) on the health of wildlife species. Since habitat quality and military activity both appear important in influencing tortoise health, implications of this finding suggests that a variety of environmental mitigation strategies could be implemented to minimize or mitigate effects of military activities by management and creation of preferred tortoise habitat.

In this project, using the integrated bioindicator approach appears to be a useful environmental management tool for assessing the relationship between the health of a TER-S such as the gopher tortoise and influential environmental factors such as military activity and habitat quality. Incorporating a variety of response variables at different levels of biological organization into the experimental design of environmental assessment studies is necessary in helping to understand causal relationships between environmental factors, organism response, and the biological relevance of such responses. Identification of those specific actions and environmental variables (stressors) responsible for injury to TER-S should reduce the uncertainty of environmental management and regulatory decisions resulting in an increased ability to predict the consequences of specific actions or activities on military ranges.

In assessing the effects of multiple environmental factors on sensitive wildlife species such as TER-S, application of suites or multiple bioindicators representing different sensitivities and specificities to stressors and levels of ecological relevance should reduce the risk of false positives (Type I error or concluding that effects are occurring when they are not), and false negatives (Type II error or concluding that effects to wildlife are not occurring when they actually are). Use of a single bioindicator or response endpoint, however, may not be adequate to reduce the probability of these types of errors. A major goal, therefore, in designing field studies to evaluate the effects of multiple environmental factors on the health or fitness of wildlife species of concern is to minimize the probability of a Type II error or a false negative (concluding that effects are not occurring when, in fact, they actually are) for the purpose of effectively protecting and managing TER-S on military ranges. For example, at Camp Shelby, environmental monitoring and assessment programs should be adequately designed to minimize the probability of concluding that various military activities such as habitat disturbance are not affecting the health and fitness of tortoises when they actually are (Type II error). Making such a false conclusion about the relationship between military activity and fitness of tortoise populations can be minimized by measuring a selective suite of multiple bioindicators that represent a range of responses at different levels of ecological relevance and also at different sensitivities and specificities to environmental factors or stressors.

Both sensitive individual level responses and longer-term integrative response indicators should be used when assessing effects of environmental factors on the health and fitness of TER-S. Integrative indices such as reproductive integrity and population fitness are structurally-related attributes which are overall indicators of environmental effects on TER-S, but these types of responses, in themselves, provide little information on the underlying mechanisms or causes of observed effects because of their relative insensitivity and slow response times to environmental stressors. Conversely, studies at the organismal and suborganismal (e.g., bioindicators and biomarkers, respectively) levels can help provide more functionally or mechanistically-related information on how stressors interact with target biological sites. The importance of organism-

level or bioindicator measures in environmental assessment studies is to provide a pivotal point through which mechanistic understanding and ecological consequences of environmental stressors and its effects on biological resources can be linked, thereby helping to identify causal mechanisms of environmental stressors.

The health and fitness of tortoise populations over the landscape of Camp Shelby is primarily related to habitat quality because habitat quality influences population size and population size is directly related to genetic fitness and therefore to overall health of tortoises. In this study we found that genetic variation of tortoise colonies or populations was greater in sites with good habitat than in sites with poor habitat and genetic diversity was also positively related to population size. Many of the study sites are affected by military land-use practices where forest habitat has been converted to areas without canopy or vegetative cover which is preferred by tortoises and has had an apparent beneficial effect on tortoise populations. To ensure that these sites continue to benefit tortoises both in the long and short term, maintenance of intervening forest habitat is requisite. An indirect benefit of maintaining surrounding forests is that tortoises may migrate into adjacent forest and, as a consequence, fewer tortoises would then occupy sites intended for military use thereby minimizing conflict of use on active ranges.

Results provided by this study suggest that some effective environmental management options are available for mitigating or minimizing potential effects due to military activity on sensitive TER-S such as the gopher tortoise. For mitigating effects of habitat disturbance due to direct or indirect effects of military activity on tortoise health, prescribed burning appears to be the most proven technique for creating and maintaining preferred gopher tortoise habitat. A management goal related to prescribed burning would be to provide a range of preferred habitat choices for tortoises by producing a mosaic of vegetation densities by altering the frequency and timing of controlled burns. Population stability and site fidelity of tortoises could be enhanced by long-term maintenance of suitable nesting and foraging habitat. Habitat manipulations that reduce canopy cover and increase available (preferred) ground forage can cause desirable shifts in tortoise population structures over time. The optimum conditions for promoting both growth of adequate herbaceous vegetation for foraging and for thermoregulation of tortoises is to have a canopy that is 20-50% open such as a mature long-leaf pine forest that is regularly maintained with prescribed fire to remove mid-story vegetation and promote growth of herbaceous ground cover. Such gopher tortoise habitats should also be managed to maintain existing genetic structure without further isolation of populations (i.e., spatial fragmentation) which could eventually result in lower genetic diversity and reduced population fitness.

The development and application in this study of methodologies and approaches for assessing effects of military activities on a representative TER-S such as the gopher tortoise should allow environmental managers at military installations to (1) help manage TER-S under conditions of ongoing military testing and training activities, (2) prioritize the management of environmental stressors or factors according to their relative importance in affecting TER-S fitness and sustainability, and (3) assist in the implementation of adaptive management strategies. The protocols and methodologies developed in this study for the gopher tortoise at Camp Shelby could also be applied to other installations where gopher tortoises occur (at least 18 in the SE-US). In addition, the quantitative methodologies developed for the gopher tortoise should be applicable, with some relative minor modifications, to other related species such as the desert tortoise because similar physiological response patterns to environmental stressors also operate

in many other cold-blooded vertebrate species. A primary contribution of this study is a more targeted ecological management strategy for military installations that can be use to (1) help mitigate the environmental stressors that are of the most concern to TER-S such as the gopher tortoise, and (2) provide guidance relative to identification and possible relaxation of those training activities that are identified to have minimal effects on wildlife species of concern.

The results of this project have provided some valuable insights as to the future directions for this study. Three important areas of future work related to this project are (1) determining the effects of cumulative environmental factors related to military activities on gopher tortoises or other related TER-S. For example, some of the military-related activities that can act as single or cumulative stressors on TER-S include habitat disturbance and fragmentation, troop use and activity, chemical (explosive residuals) exposure, noise, invasive species (can be both military and nonmilitary), and fire (control burns), (2) technology demonstration using gopher tortoises or similar species as proof-of-principle at two other military installations. Environmental managers at both Fort Stewart and Fort Benning have endorsed and are supportive of this type of study, (3) specific studies related to landscape population genetics which would determine the role and importance of habitat quality and fragmentation in the health and population fitness of gopher tortoises.

OBJECTIVES

The primary objective of this project is to develop and apply a bioassessment approach that can be used by environmental resource managers at military installations to evaluate the fitness or health of Threatened, Endangered, and at-risk Species (TER-S) through the measurement of a selected suite of sensitive and rapidly-responding bioindicators. This project was in response to SERDP SON number CSSON-04-4 related to the use and application of a physiologically-based framework for assessing the effects of military activities on TER-S. This study is primarily related to the SON through its emphasis on chronic or sublethal (i.e., physiological) responses of TER-S to military activities.

This study takes an empirical, proof-of-concept approach to evaluate if these sensitive and rapidly-responding bioindicators can be used by DoD resource managers as a practical and cost-effective tool for evaluating the health status of TER-S. Stated as a hypothesis, the primary research objective is: "bioindicators, which are sensitive, rapidly-responding, and early-warning response measures to environmental factors, can be used to assess or predict the fitness and health of TER-S at military sites." A suite of bioindicators of stress, developed in this study and calibrated against individual and population-level fitness endpoints, is used to assess the health of TER-S relative to environmental factors such as habitat quality and level of military activity. This bioassessment technique should enable the military to more rapidly and cost-effectively determine if activities are compromising the health or status of at risk wildlife species.

The main objective during the first year (FY 05) of this project was to design the experimental field studies for sampling gopher frogs (*Rana capito*) at Eglin AFB and for sampling gopher tortoises (*Gopherus polyphemus*) at Camp Shelby. The second year of study (FY 06) focused on construction and installation of sampling devices at both military installations, sampling of gopher tortoises at Camp Shelby, MS, and sample processing and analysis of bioindicator samples. During the third year of this project (FY 07-08) the emphasis was placed on conducting various types of habitat measurements and analysis including special studies related to landscape population genetics and reproductive competence investigations to evaluate the relative importance of habitat quality and military activity on the health and condition of gopher tortoises at Camp Shelby.

Due primarily to the exceptional drought in the Southeastern US over the entire duration of this project during which frogs could not be collected at Eglin AFB, the bioindicator studies originally planned for gopher frogs had to be discontinued. The remaining SERDP funds originally earmarked for this effort (sampling of frogs, processing and analysis of samples, etc) were redirected toward extension of some additional gopher tortoise studies at Camp Shelby. Results of our original studies on gopher tortoises at Camp Shelby indicated the need to conduct further investigations that could help identify or explain the relative poor condition and fitness of tortoises at Camp Shelby. Since habitat and military activity both were identified as important in influencing the health and condition of tortoises at Camp Shelby, these additional studies focused on what specific aspects of military activity and habitat were the most important in influencing the survival and health of these tortoises. These are important issues to address relative to the implications for environmental mitigation and management strategies, not only at Camp Shelby,

but at other installations which also deal with similar issues related to TER-S. As further investigations into the importance of military activities and habitat quality on tortoise health and fitness at Camp Shelby, additional studies were also conducted on food habits and diet quality, reproductive fitness, and landscape population genetics, the results of which are provided in this final report.

BACKGROUND

The DoD manages approximately 25 million acres of land in the United States, much of which has been sheltered from extensive development pressures and large-scale habitat loss (DoD 2002a). In addition, TER-S are present on DoD installations in greater abundance on a per area basis than on most other federal lands (Leslie 1996, Tazik and Martin 2002). The DoD faces a difficult challenge in balancing the need to conduct essential military training and testing activities while continuing to address stewardship responsibilities for the rich variety of natural resources it manages (DoD 2002a & 2002b, GAO 2002). At risk and endangered species management on military lands can be particularly challenging, with some federal laws and regulations requiring extensive TER-S evaluations and potentially lengthy formal consultations with federal and states agencies [e.g. Biological Opinions (BO) as part of Section 7 of the Endangered Species Act process]. Laws that strongly affect TER-S considerations at military installations include: (1) the Sikes Act of 1960, (2) the Endangered Species Act (ESA) of 1973, and (3) the Sikes Improvement Act of 1997 (GPO 2003). The presence of TER-S on military lands has led to training restrictions at many installations (Guertin 2005). As a result of these restrictions the Army has, for example, mandated that assessing impacts of military operation on TER-S (section 4.6a of the DOA pamphlet 420-7) is the highest-ranked Army requirement in the conservation pillar of the Environmental Quality Technology Program. Studies conducted under Requirement 4.6a are committed to assessing impacts of military training activity on TER-S. Given the many and complex issues associated with TER-S on military lands, DoD environmental managers need all the tools, training, and resources available to successfully manage TER-S resources in a sustainable manner.

This SERDP project has as its main goal the development and application of an integrated and multivariate bioindicator (physiological) approach for assessing effects of military activities and other environmental factors on the health and fitness of a representative TER-S at Camp Shelby, MS. Advantages of using multiple bioindicators to assess the health and fitness of wildlife species are that bioindicators serve as early-warning response indicators which are more sensitive, specific, rapidly-responding, and often less expensive than most traditional bioassessment approaches (Adams 1990, Adams 2002, Adams et al. 2002, 2003). Two of the major limitations of most traditional approaches for assessing effects of environmental factors on TER-S are (1) assessment of effects on TER-S often involve population census or demographic studies which can be highly variable, expensive, and often labor intensive and, by definition, TER-S are relatively rare and information generated from such studies can result in unreliable estimates of population fitness, and (2) if, in fact, observable population level effects can be determined by conventional assessment approaches, the probability of implementing effective remedial or environmental management action plans after the fact is compromised because irreversible damage has probably already occurred at the population level. Using a selected suite of sensitive and rapid- response bioindicators of health within a framework of a weight-of-evidence approach, however, should allow environmental resource managers at DoD facilities to predict and assess potential effects of military activities before the fitness of TER-S is severely compromised. Such studies would also provide valuable information relative to evaluating if current military activities are consistent with maintaining healthy and sustainable populations of TER-S on active ranges.

The gopher tortoise, *Gopherus polyphemus*, occurs on at least 18 military installations in the SE-U.S. (Wilson and Mushinsky 1997) and this species has been identified as a TER-S of priority research and interest for the Army. Over the past decade, only the red cockaded woodpecker (*Picoides borealis*) has received more attention as the gopher tortoise in terms of research, management, and protection of a TER-S on military reservations. The gopher tortoise is one of the Army's highest TER-S of concern which has the potential for restricting training on military installations (Guertin 2005), including Camp Shelby, MS where this study was conducted.

The gopher tortoise ranges from southeastern Louisiana to southeastern South Carolina and it is federally protected as a threatened species west of the Mobile and Tombigbee rivers in Alabama. In Florida and Georgia it is State listed as a threatened species and in South Carolina and Mississippi (Camp Shelby) it is State listed as an endangered species. In addition to its wide distribution and occurrence at military sites in the southeastern U.S., several life history characteristics of this species such as herbivory, limited home range, and long life span renders it particularly vulnerable to environmental factors such as habitat disturbance and activities resulting from both military and non-military sources.

One of the keystone species in the upland systems of the SE-U.S. is the gopher tortoise (Eisenberg 1983). A keystone species is one that is important relative to the structure and function of ecological communities, and through its activities, is responsible for a significant portion of the energy flow in a system. Burrows excavated by gopher tortoises may contain the richest species diversity of all North American animal burrows with more than 360 species recorded (Jackson and Milstrey 1989). Burrows serve as hiding places, nesting sites, or overwintering dens for many obligate and facultative commensals (Guyer et al. 1996), many of which are legally protected (Schwartz and Karl 2006)

The integrated bioindicator approach is an effective method to determine and evaluate the effects of various environmental stressors such as habitat disturbance and military activities on the health of a TER-S such as the gopher tortoise. This approach involves measuring a suite of biological responses including the sensitive and rapidly-responding biomarkers and the more integrative and ecologically-relevant bioindicators. The relationship between biomarkers or responses measured at the lower levels of biological organization (biomolecular, biochemical, etc) and the more ecologically-relevant bioindicators of effects are shown in Fig. 1. These suites of biological measurements represent a gradient of biological responses from the early warning and sensitive biomarkers to the more ecologically-relevant but less sensitive bioindicators. Biomarkers of exposure to environmental stressors generally include biomolecular and biochemical responses and bioindicators of longer-term effects include organism level, reproductive, and population responses (Adams 1990, Adams 2002). There are several benefits which result from incorporation of both biomarkers of exposure and bioindicators of effects in this which links sublethal (physiological) response indicators to reproductive and population-level endpoints. In general, biomarkers are used to indicate exposure to a stressor, and bioindicators, mainly because of their integrative nature, are used as indicators of exposure effects at higher levels of biological organization. Because biomarkers are stressor-sensitive and rapidly-responding endpoints, they can be used to identify the mechanistic basis of possible causal relationships between stressors and effects (Fig. 1). They can also be used to help identify

the source of an environmental stressor or determine if organisms have indeed been exposed to a specific stressor or a group of similar stressors such as explosive residuals. The multivariate bioindicator approach for assessing effects of environmental stressors on wildlife is conceptually similar to that used by the medical profession to diagnose the health of human patients. In human subjects, blood chemical profiles and other medical procedures are performed, and the results compared to standardized norms.

Within the past two decades biomarkers along with bioindicators have been used successfully as research tools to address a variety of issues related to chronic or sublethal effects of environmental stressors on ecological systems. Some of the many notable examples that have addressed the use of biomarkers and bioindicators in field studies relative to assessing the effects of environmental stressors on biota and ecosystems include Adams and Greeley (2000), Adams et al. (2002), Adams et al. (2003), Adams (2005), Adams et al (2005), Depledge (1994), Beliaeff and Burgeot (2002), Sarkar (2006), Handy et al. (2003), Hyne and Maher (2003), Galloway et al. (2004), and Triebskorn et al. (2001).

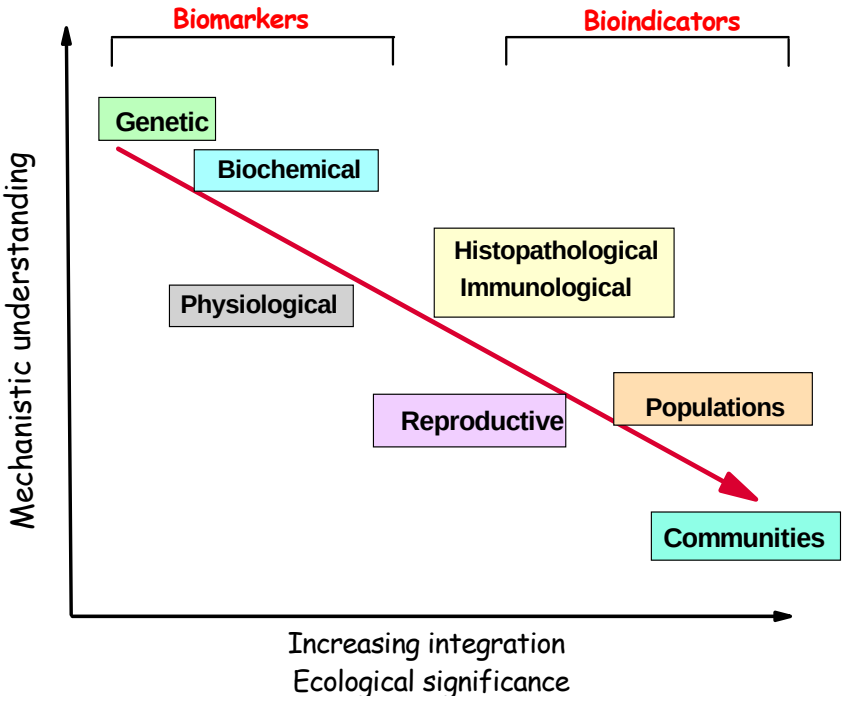

Figure 1. Relationship between biomarkers and bioindicators relative to their value in understanding the relationship between mechanisms of exposure (biomarkers) and ecological significant effects (bioindicators) in biological systems.

TECHNICAL APPROACH

The original design of this project included the study of gopher tortoises at Camp Shelby, MS and gopher frogs at Eglin AFB, Florida. Due to the extreme drought situation in the Southeastern US over the past three years, frogs could not be collected at Eglin AFB (see Objectives Section), however, the experimental design studies including the habitat and chemical characterization components were completed. Consequently, most of the effort on this project has been focused on the gopher tortoises at Camp Shelby. This technical approach section is divided into the gopher tortoise studies at Camp Shelby and the gopher frog studies at Eglin AFB, the latter which includes the experimental field design and the habitat and chemical characterization studies.

The sustainability of wildlife populations on military ranges can be affected by both habitat quality and other environmental factors or stressors such as exposure to explosive residual compounds. Assessment of environmental stressors to wildlife on military ranges should consider physical habitat disturbance in addition to other possible environmental stressors such as contaminants in order to distinguish habitat-related effects from these other potential effects on wildlife (Efroymson et al. 2009). Thus, the experimental design of this project incorporates both a habitat effects component and a military effects component.

Gopher Tortoises-Camp Shelby

Experimental field design

The experimental field design for the gopher tortoise studies at Camp Shelby consists of three major components, (1) site selection and design, (2) habitat characterization, and (3) chemical (explosive residuals) characterization.

Site selection and design

The basic experimental field sampling design for the gopher tortoise study at Camp Shelby consists of 6 experimental treatments, each with 3-4 replicate sites including areas that are characterized by (1) high military activity and good habitat, (2) low military activity and good habitat, (3) low military activity and poor habitat, (4) no military activity and poor habitat, (5) no military activity and good forested habitat (gopher tortoise refuge), and (6) no military activity and good ruderal (grass type) habitat (Fig. 2). Good habitat is defined as either (1) cleared areas on Camp Shelby such as firing points that are maintained as grass and short ground-level vegetation (ruderal vegetation) or, (2) forested areas where the tree canopy is 50% or more open allowing sufficient light at ground level to support low-level vegetation such as grasses, herbs, and legumes. This later type of habitat is typically characterized by mature stands of longleaf pine (*Pinus palustris*) with minimal mid-story vegetation. Locations of sample sites representing the six treatment categories for gopher tortoises at Camp Shelby are shown in Fig. 3. Aerial images of some representative sample sites are shown in Fig. 4 including a high activity-good habitat site, a low activity-poor habitat site, and a demolition range.

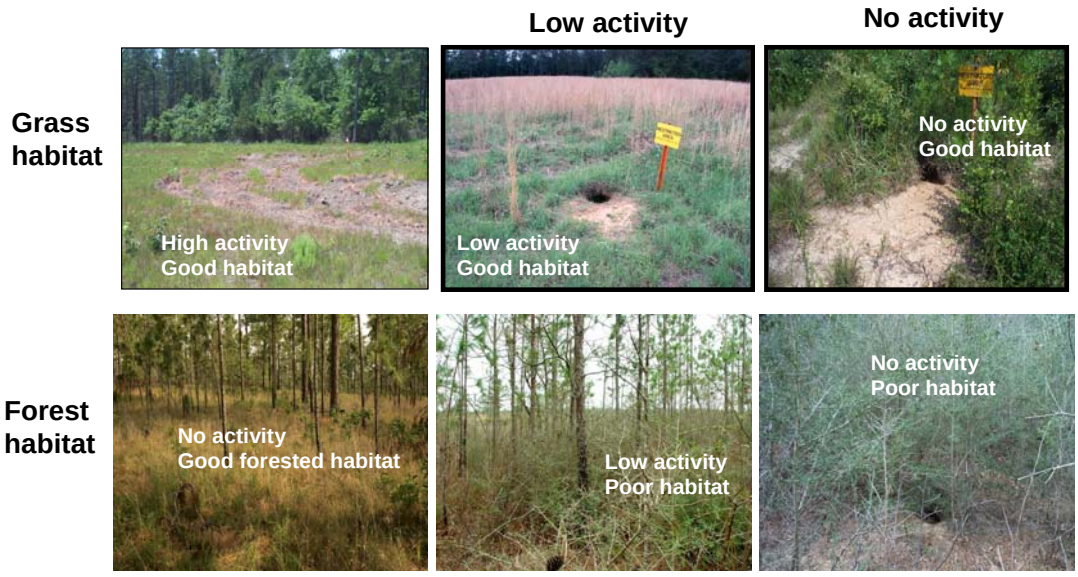

Figure 2. Examples of the six experimental treatments for investigating the effects of military activity and habitat quality on the health of gopher tortoises at Camp Shelby.

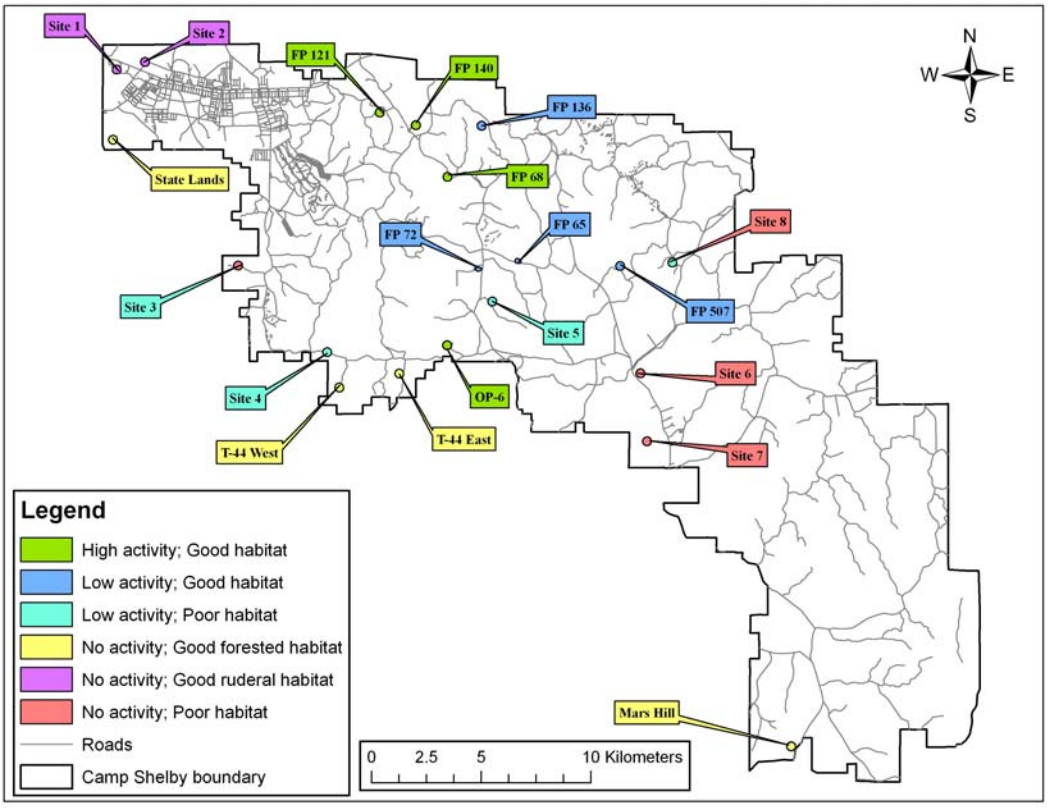

Figure 3. Locations of sample sites representing the six different experimental treatments where gopher tortoises were sampled at Camp Shelby. Each color-coded treatment is represented by 3 replicated sites.

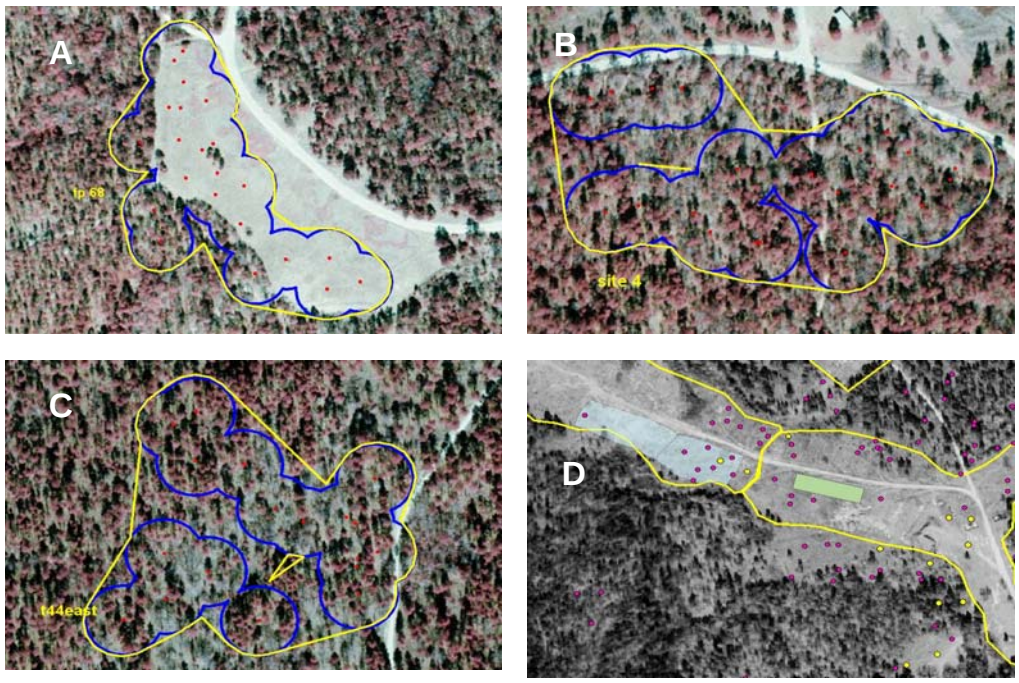

Figure 4. Aerial images of representative experimental treatment types at Camp Shelby for the gopher tortoise including a high activity-good habitat firing point (4A), a low activity-poor habitat site (4B), a low activity-good habitat area (4C), and the OP-6 demolition range which is a high activity (high energetic compounds) and good habitat area. Yellow borders are the 30m foraging home range boundaries (radii) of the gopher tortoise burrows. Detailed chemical and habitat characterization studies were conducted within these boundaries.

Level of military activity at each sample site was determined by assigning a high, low, or no activity status based on a quantitative ranking system using three major assessment criteria including (1) the number of rounds of munitions used at a site or firing point, (2) the number of ground troops utilizing a site, and (3) noise levels based on model projections of decibel isopleths over different areas of the base (Table 1). Munitions use at a particular site was based on a subjective ranking of 0-3 based on the number of rounds fired. Troop use was also rated from 0-3 based on the number of troops utilizing a particular firing point, and noise was rated from 1-3 based on the decibel isopleths at a particular site. For each site, the rankings were summed over munitions use, troop use, and noise, and each sample site was then designated either as a high, low, or no (reference) activity area depending of the values and segregation of the final composite scores. Data on munitions use (primarily 155m howitzers) along with troop use and activity were obtained from range control operations at Camp Shelby and were averaged over a two year period. These numbers represent the real time information reported to Range Control by the range commanders. Even though this data may not be absolutely accurate regarding the actual number of troops that may have used a site or firing point at a specific time, they do however reflect a pattern or trend for long term use over a site and represent, therefore,

high, intermediate, or low military activity over the two years represented by this data. The purpose of the troop and ammo use data was simply to help designate sites as to their relative status of military activity, and no statistical analysis was necessary. Even if the troop use data were a few hundred individuals off at some sites for a particular reporting period, this, in itself, would not have overly influenced the final military activity designations of a site regarding its high, low, or intermediate long-term designation. In other words, the actual numbers for troop use at each site is not as important as the relative numbers that determined which particular sites fall into the three military use categories (Table 1).

Table 1. Assessment of military activity at various sites at Camp Shelby. Military activity was assessed by determining the amount of munitions used at each site, troop use at a site, and noise levels.

Firing point	Ammo use	Ammo rating[1]	Troop use	Troop rating[2]	Noise rating[3]	Composite score	Military activity
121	476	2	2952	2	3	7	High
71	1244	3	6352	2	2	7	High
91	413	2	24021	3	1	6	High
68	317	2	5335	2	2	6	High
65	0	0	6822	2	2	4	Intermediate
101	0	0	1739	1	3	4	Intermediate
62	0	0	4537	2	2	4	Intermediate
64	0	0	3865	2	2	4	Intermediate
136	78	0	1213	1	1	2	Low
99	0	0	350	1	2	3	Low
507	0	0	0	0	3	3	Low
97	0	0	671	1	2	3	Low

[1]Ammo rating
rounds > 1000 = 3
300-500 = 2
100-200 = 1
<100 = 0

[2]Troop use rating
troops > 10,000 = 3
2000-10,000 = 2
100-2000 = 1
<100 = 0

[3]Noise rating
Based on noise decibel contours obtained from Range Control at Camp Shelby

Habitat characterization

Physical modification of habitat from all training activities is the primary disturbance affecting vertebrate populations on military installations. (Demarais et al. 1999). Thus, one of the main priorities in the experimental design of this study was on habitat-related factors.

The primary purpose of the vegetative analysis is to evaluate possible relationships between tortoise health and the type and quality of the habitat at each sample site. Assessment of habitat quality and the level of habitat disturbance at each sample site was determined using GIS technology in combination with direct plot-based measurements of key habitat and vegetation metrics. Habitat disturbances of potential consequence to gopher tortoises included assessment of land management related impacts (e.g., type and extent of logging, use of controlled fire or absence of fire, type and frequency of range clearing and/or vegetation restoration techniques), more direct military impacts to habitat (e.g., vegetation trampling from tracked and untracked vehicle use, soil compaction, or development of road and other infrastructure that may limit tortoise movement), or even natural disturbances such as fire and tree blow-down from hurricanes. Mechanistically, habitat disturbance can modify the availability and quality of forage, affecting tortoise nutrition and energy dynamics (Jodice et al. 2006, Oftedal and Allen 1996), disturb or compact soils necessary for burrowing and egg laying, encourage invasive species that prey on hatchlings (e.g., fire ants), and create barriers to movement of individuals between burrows or colonies (e.g., dominance of shrubby undergrowth, bare ground, or roads and other infrastructure). The gopher tortoise GIS Database at Camp Shelby contains accurate and up-to-date survey data of active and inactive gopher tortoise burrows associated with land cover types, range boundaries, physical characteristics (soils, hydrology, topography), and distance from roads and other military infrastructure.

Detailed vegetation survey data was obtained by on-the-ground surveys using direct plot-based vegetative assessments (Figs. 5 and 6). Direct plot-based assessment of vegetation is an effective method for evaluating the effects of military disturbance (Dale et al. 2002, Prosser et al. 2003). At Camp Shelby, the following habitat-related metrics were determined for a series of plots located near burrows and colony forage areas: plant species richness, percent species cover, percent bare ground and litter, the number of fire ant mounds, and canopy cover and basal areas of trees (Fig. 6). The quantity and diversity of plants within the tortoise forage areas as determined by plot-based measurement as well as qualitative vegetation surveys along the tortoise trails from each burrow were determined. Vegetative analysis was based on habitat assessment surveys conducted (1) near the burrow entrance (within 1.5m radius of burrow) where tortoises spend about 50% of their time, (2) at a 30m radius of the burrow where tortoises spend approximately 90% of their time foraging and nesting, and (3) the vegetative buffer zone represented by a 200m radius from the burrows (Figs. 5 and 6). McRae et al. (1981) reported that the feeding radius of gopher tortoises is 8-13m in a circular or elliptical pattern around individual burrows.

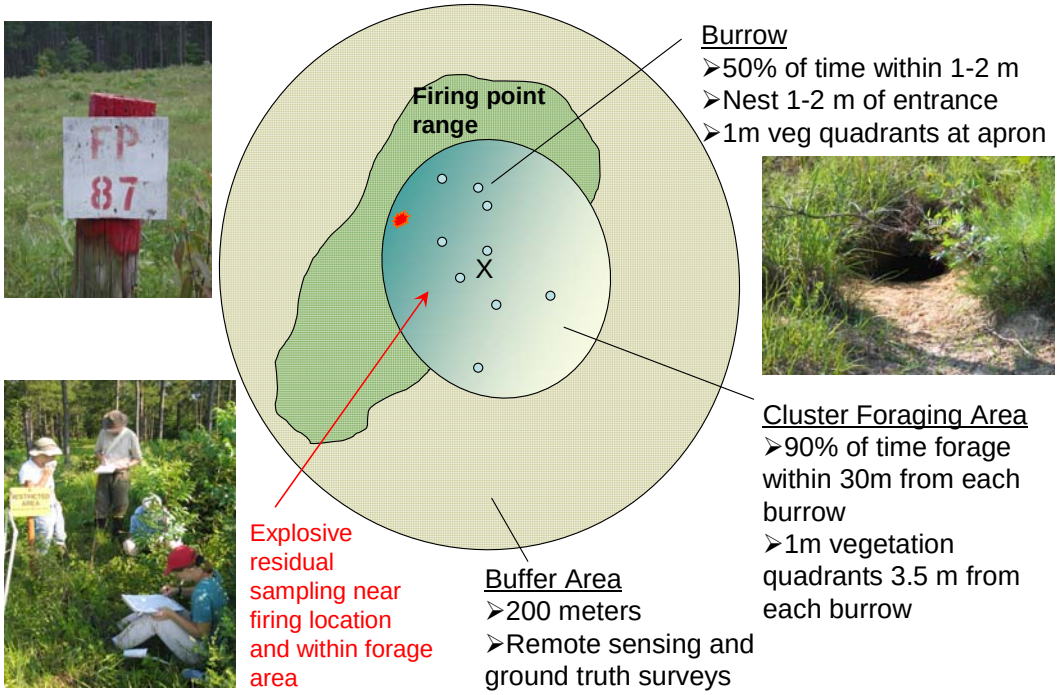

Figure 5. Basic field procedures for characterizing the habitat of gopher tortoises at Camp Shelby. To thoroughly characterize the environment of the tortoise, various habitat and habitat disturbance metrics were measured including those in the immediate burrow area, 30m foraging zone, and 200m buffer zone.

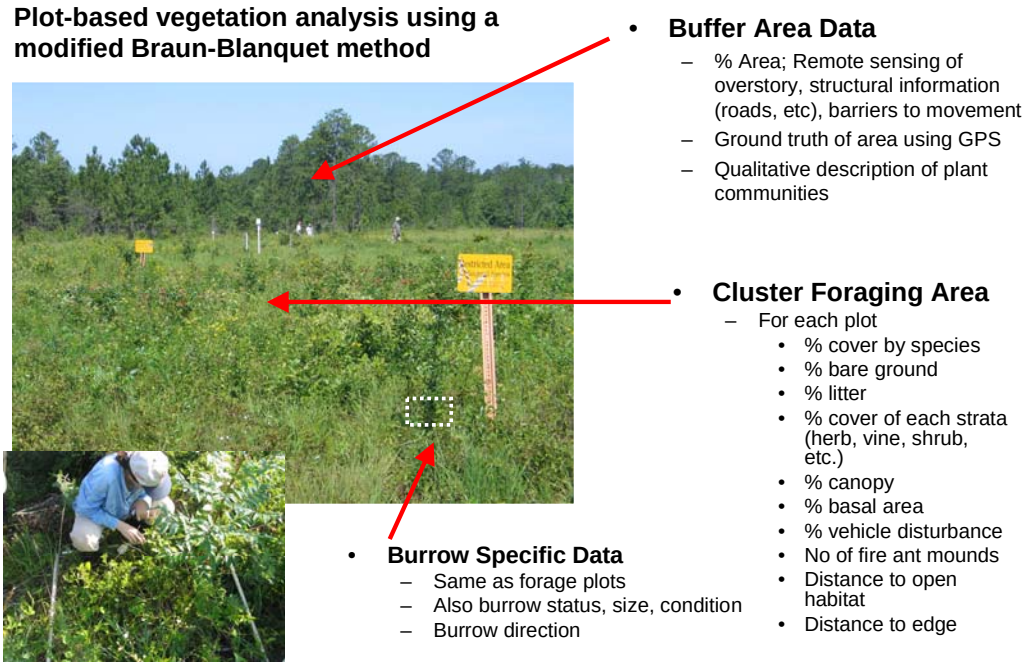

Figure 6. Specific habitat metrics measured at each of the gopher tortoise vegetative zones including the 1.5m radius area of the burrow, the 30m radius area of the burrow, (cluster foraging area), and the 200m buffer area.

Each data record for the habitat quality and vegetative assessment was referenced by a unique burrow code. After field collection, the data were related to an existing geo-referenced burrow information database available from Camp Shelby. This procedure enabled each data record to be associated with a specific location that was identifiable on a Geographic Information System (GIS). This process involved data cleaning and validation which was conducted in collaboration with Camp Shelby personnel. Similarly, field data collected for the 200m buffer radius from each burrow usually included larger vegetation such as pine and hardwood trees, mid-story trees, and tall shrubs. These data records included on-site GPS measurements that enabled it to be plotted on a GIS. All the field based data were summarized by site to better define the habitat characteristics for all sites and treatments.

Once the location of data records for the field survey information (collected at 1.5m of the burrow, 30m from the burrow, and the 200m buffer area) were accurately defined, other ancillary data considered important for characterizing the habitat of these sites and treatments were obtained from Camp Shelby staff. Such ancillary information included remote sensing-based data such as historical and recent aerial photographs, land cover data, soil types, fire history, location of streams, other water bodies, roads etc. Combining field-based data (such as vegetative types and cover) and remote sensing information helps to better define and characterize a site and also provides addition information that could not be derived from field survey information alone. Metrics such as percentage of pine forest, length of roads, percentage of poor habitat, percentage of edge within a site etc. can be derived from the combined database. Further, the remote sensing-based land-cover data aids in providing a holistic characterization and evaluation of the entire environment of the tortoise and identifies potential barriers to

movements and foraging and also habitat corridors for movement. This information was used along with the tortoise health data to evaluate the relative importance of habitat, military activity, and other physical features of the environment on tortoise health and to help explain the observed differences in tortoise health among sites and treatments.

Chemical characterization

Chemical characterization studies were conducted at each of the experimental field sites at Camp Shelby according to the methods of Jenkins et al. (2005) to determine the levels of explosive residuals in soil (Fig. 7). Working with Tom Jenkins and Alan Hewitt from ERDC/CRREL, soil samples were collected from Camp Shelby at several firing points, at an ordnance demolition range (OP-6), and at some of the reference sites. Each sample site or firing point was divided into 2-3 equal-sized grids and within each grid a composite sample was taken by combining approximately 100 increment soil samples obtained at evenly spaced locations within the grid. Increment samples were taken with a coring tool and the top 2cm of material (soil and vegetation) retained. Thus, levels of explosive residuals in the soil for each treatment replicate were based on 200-300 increments or subsamples which were composited from each grid for analytical analysis.

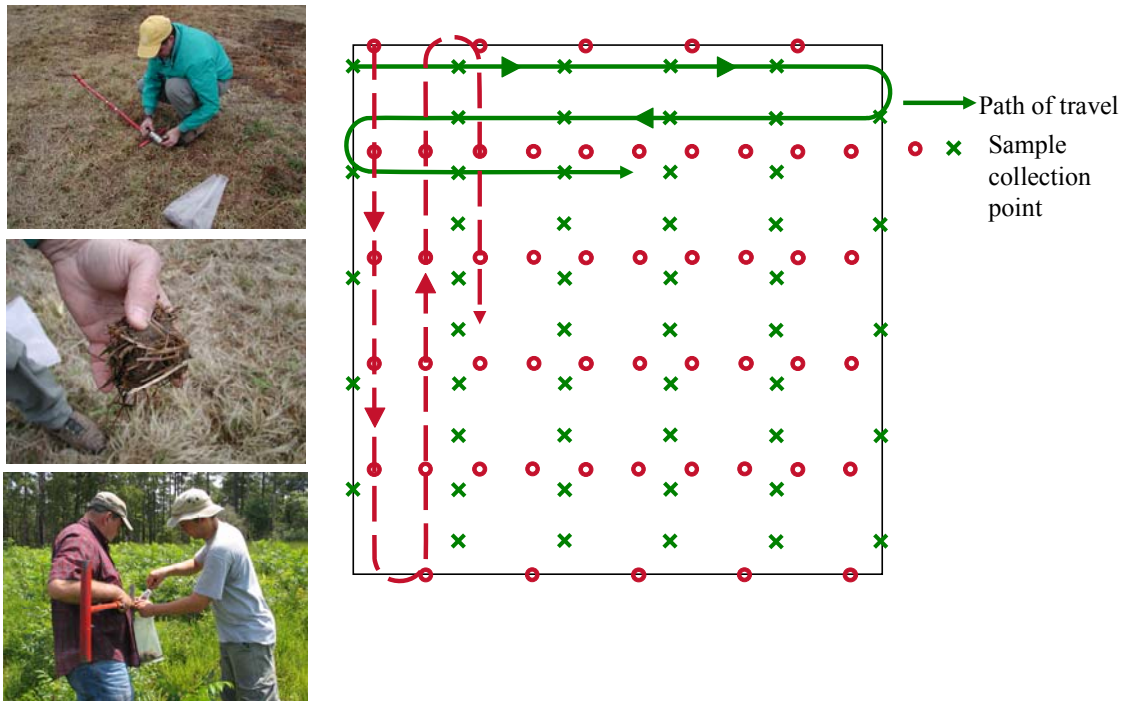

Figure 7. Basic procedure for conducting the chemical characterization studies at Camp Shelby and Eglin AFB. Explosive residuals in soil were assessed by collecting replicate (green Xs and red circles) soil core increments within a 50m^2 or a 100m^2 grid at 5m or 10 meter intervals, respectively.

Biological sample collection and processing
At each of the 20 sample sites (three treatments had 4 replicate sites, 2 treatments had 3 replicate sites, and 1 treatment had 2 replicated sites), live traps were placed at the entrance of each active burrow. Burrow camera surveys were first conducted using underground scoping techniques to determine which burrows on each of the 20 sample sites harbored gopher tortoises. Active burrows, or those containing a tortoise, were identified to confirm that a tortoise was present before a live trap was placed at the entrance. Traps were placed at each burrow in late afternoon and checked at mid-morning and mid-afternoon each day after that until the tortoise was captured. Population estimates for each site and treatment type were obtained based on the catch statistics and number of active burrows on the site. This population estimate included the number of tortoises that were actually captured at each site along with the burrows that were identified as active from the camera burrow scoping surveys.

Immediately upon capture, blood samples were taken from each tortoise from the brachial vein using heparinized syringes, and the blood samples and tortoises then transported to a central processing facility (laboratory) on the base. At the laboratory, basic morphological information was taken on each tortoise and recorded on field data sheets including total weight, total length, plastron length, thoracic height, and width of anal scutes. In addition to morphological measures a general health assessment, using external features and parameters, was conducted on each tortoise following the guidelines of Berry and Christopher (2001). The sex of each tortoise was also determined and, for females, additional procedures were performed to determine their reproductive condition. Females with eggs were given an injection of oxytoxin to induce egg deposition, and the deposited eggs were then placed in trays in a constant temperature incubator in the laboratory for the purpose of ultimately determining hatching success and hatchling survival. Females that did not respond to the oxytoxin injection were transported to a local veterinarian and full-body radiographs taken. The radiographs were used to determine clutch size (number) and egg quality (size) by measuring the short and long dimensions of each egg shown on the radiograph.

Sample analysis
In the laboratory, blood samples were processed and the immunological analyses were conducted on site. Each blood sample was processed and divided into several aliquots and prepared for a variety of analyses to be performed at a later time including (1) hematology (blood smears for differential cell counts and basic hematological analysis), (2) immunological and corticosteroid stress hormone analysis including the bacterial killing assay which was performed on site, (3) serum chemistry profile analysis, (4) reproductive hormones, (5) biomolecular analysis including indicators of DNA damage and oxidative stress, (6) population genetics, and (7) upper respiratory tract disease (URTD).

Hematology - The hematocrit or percentage of whole blood composed of red blood cells was determined by the microhematocrit tube method. Blood cell differentials or the complete blood count (CBC) was assessed by smearing two drops of blood on a microscope slide, drying, and counting the number of leucocytes (white blood cells) and the different types of leucocytes including lymphocytes, monocytes, eosinophils, basophils and heterophils. Cell differential counts were performed on two male and two female tortoises from each sample site.

Blood chemistry profile -Whole blood was centrifuged and the remaining serum was transferred to separate vials, labeled, and frozen for later analysis. The following analyses were performed on serum samples using a standard clinical blood analyzer to generate a blood chemistry profile for each tortoise: (1) indicators of electrolyte homeostasis including phosphorus, calcium, magnesium, sodium, potassium, chloride, bicarbonate, anion gap, NA/K ratio, and osmolality, (2) an indicator of carbohydrate metabolism and general stress (glucose), (3) indicators of protein metabolism (total protein, albumin, globulin, alb/glob ratio), and (4) indicators of tissue/organ dysfunction (urea nitrogen, uric acid, bilirubin, alkaline phosphatase, aspartate aminotransferase or AST, lactate dehydrogenase or LDH, creatinine kinase, and gamma-glutamyl transferase or GGT).

Immunological response: the bacterial killing assay - This procedure measures the ability of the immune system to destroy pathogens in the blood using a bactericidal or phagocytic assay. Whole blood collected from the field was diluted to 1:50 in CO_2-independent media and *E. coli* (ATCC 8739; Microbiologics, USA) was diluted to 1:1000 using sterile phosphate buffered saline (PBS). A total of 140µl of diluted blood was mixed with 10µl of diluted bacteria. A total of 50µl of this combined blood/bacteria solution was spread onto individually labeled trypticase soy agar plates (BD Diagnostic systems, USA) at 0 and 60 minutes post-mixing. Two control plates of CO_2-independent media with diluted bacteria (no blood) were used. All plates were incubated at 37°C for 24 hours. Colonies of *E. coli* were then visually counted and recorded. The following equation was used to calculate an index of the bactericidal (phagocytic) ability of the blood:

$$BKAindex = -1 \times \left(\frac{bka60 - bka0}{bka0} \times 100 \right) - \left(\frac{control60 - control0}{control0} \times 100 \right)$$

where: bka60= blood/bacterial mix for 60 mins. prior to plate culture,
bka0= blood/bacteria mix zero mins. prior to plate culture,
control60= diluted bacteria only for 60 mins. prior to plate culture,
and control0=diluted bacterial only for zero mins. prior to culture

This index assigns large positive values to tortoises with increased phagocytic activity against the bacteria, and negative values to tortoises whose blood had little or no phagocytic activity, which in some cases resulted in an increase in bacterial colonies due to growth instead of elimination during incubation. By incorporating the control measures, this index also takes into account bacterial die-off within each assay that may have been caused by factors unrelated to the blood's bactericidal ability.

Adrenal stress hormone response - The glucocorticoid hormones or corticosterones are good indicators of chronic or sublethal stress in animals (Rice and Arkoosh, 2002). Baseline cortisol was measured in the blood of tortoises collected from each site. In the laboratory, male tortoises also received an IP injection of adrenocorticotropic hormone (ACTH) to stimulate the adrenal cortex to produce cortisol (e.g., the ACTH challenge test). Four hours following injection, a small blood sample (200 ul) was taken from each male tortoise and cortisol was again measured. The difference between the initial (baseline) cortisol levels and that produced from the ACTH

challenge is a measure of the ability of the immune system of the tortoise to respond to environmental stressors, with a high response indicating a healthy immune system. A challenged immune system would be indicated by a depressed response to ACTH.

Upper respiratory tract disease (URTD) - An aliquot of the blood (serum) collected from tortoises in the field was sent to the Department of Infectious Diseases and Pathology, College of Veterinary Medicine, University of Florida to analyze for the presence of *Mycoplasma agassizii* antibodies, which is the etiological agent of chronic upper respiratory tract disease in the gopher tortoise (Brown et al. 1994). An enzyme-linked immunosorbent assay (ELISA) was used for the detection of *M. agassizii*-specific antibodies in the tortoise and was developed with a monoclonal antibody with specificity for the tortoise immunoglobulin light chain (Brown et al. 1999).

Special studies

Due primarily to the exceptional drought in the Southeastern US during the entire duration of this project, the bioindicator studies originally planned for gopher frogs at Eglin AFB were discontinued at the end of the second year of this project. The remaining SERDP funds which were originally earmarked for this effort were redirected toward extension of some additional gopher tortoise studies at Camp Shelby including food habitats/diet quality, landscape population genetics, and reproductive studies of tortoises.

Food habits/diet quality

A diet quality and food habitats study was conducted for over 100 tortoises which were collected during the primary sampling of tortoises in 2006. Prior to analysis, tortoise scats were stored in a –20°C freezer. Before analysis, scats were defrosted for 48+ h at 4°C and then weighed. Scats were placed in a large petri dish or dissecting pan, and components were individually separated under a Zeiss Stemi 2000C dissecting microscope using forceps and dissecting needles. If the scats were relatively hard, dry, or sandy, water was added to the samples to clean and float out parts. Putatively identifiable components were placed and stored in one bottle of 70% ethanol, and unidentifiable parts, digested material, and leftovers were placed in a different bottle of 70% ethanol.

For identification and determination of abundance for major food items, putatively identifiable material was placed in a dissecting pan, foliage was sorted into species, and seeds were separated. Approximate amount of each species (or category of species) was estimated by using a 5 × 2 grid in the pan. If smaller amounts of some species or categories were present, but hardly noticeable, they were given a default value of 0.1%.

Foliage and seeds were identified by comparison with herbarium material at the University of Southern Mississippi herbarium. A list of plants collected from each sample site at Camp Shelby (vegetative surveys), a published local flora (Rogers 1977), and previous studies of gopher tortoise diet (Birkhead et al. 2005, Macdonald and Mushinsky 1988) served as a baseline of taxa that were likely to be encountered. Martin and Barkley (1961), Montgomery (1977), and the online U.S. Department of Agriculture (USDA) plants database (http://plants.usda.gov/) were used to aid in seed identification. All sample material is stored in the Spirit Collection at the University of Southern Mississippi, Hattiesburg, MS.

Population genetics

A population genetics study was conducted to determine genetic variation of tortoises across Camp Shelby, how this genetic variation is spatially distributed, and what factors best explain the distribution of this genetic variation. Factors included in the analyses to interpret genetic variation included geographic distance and landscape features between sites, gopher tortoise natural history and spatial ecology, and land use history of the base. This study was based on blood samples from 340 adult tortoises collected at 34 sites across the entire base. Approximately 50% of these tortoises were collected during our primary 2006 sampling period, and blood samples from the other tortoises were obtained from historical samples collected back to the late 1990s. Such a study allows us to use genetic data to evaluate the effect that landscape features have on tortoise distributions and movements across Camp Shelby. The primary objectives of this study were, therefore, to (1) identify genetic variation across the base and within tortoise colonies; (2) determine which factors are important in affecting genetic variation, including habitat quality, military activity, population size, and sample size; and (3) attempt to identify genetic partitioning among populations across the base (i.e., population structuring) and how this relates to landscape variables, including geographic distance, landscape features, and other possible influential biological and environmental variables.

Whole genomic DNA was isolated from all blood aliquots using a Qiagen DNEasy® kit and protocol. We optimized conditions for polymerase chain reaction (PCR) of 12 microsatellite loci— nine microsatellite loci developed by Schwartz et al. (2003) and 3 loci developed in desert tortoises by Edwards et al. (2003). All 3 loci from Edwards et al. (2003) were excluded because two had no variation (i.e., only a single allele was detected) and the other appeared to be heterozygous for every individual, which is not possible if the locus being amplified is actually a microsatellite DNA region. Therefore, the final population genetic analyses were based on the 9 loci developed by Schwartz et al. (2003), which were amplified for each tortoise using PCR. PCR conditions during amplification followed Schwartz et al. (2003). Tortoises were genotyped for each microsatellite DNA locus first by collecting data on an ABI 310 Genetic Analyzer (Applied Biosystems, Inc.) by pooling samples of PCRs for three loci per individual using different fluorescently labeled primers for each locus. Allele sizes were then scored using Genescan v. 3.2 (Applied Biosystems, Inc.). In total, 340 tortoises from 34 sites were genotyped for the 9 loci. All genetic analyses were performed using FSTAT v. 2.9.3. (Goudet 1995, 2002). Prior to conducting the primary analyses, data were examined for linkage equilibrium between all pairs of loci in each sample using a log-likelihood ratio G-statistic and tested for Hardy Weinberg equilibrium (HWE) within each sample using global tests with 10,000 randomizations. Alpha was adjusted using Bonferroni corrections. No linkage disequilibrium was found among the nine loci but significant departures from HWE were detected. Therefore, where applicable, genetic analyses were performed that did not assume HWE.

The genetic analyses described below were performed within and between the individual study sites. Additionally, analyses were performed within and between groups of sites (hereafter referred to as "colony groups"). These colony groups were defined *a priori* based on the distribution of tortoises across the base, however, it is possible that the individual sites were part of larger populations. Delineation of colony groups were based on (1) the proximity of tortoise burrows within and between our sites, (2) landscape features, and (3) known gopher tortoise spatial ecology on Camp Shelby. Sites were clustered into 9 colony groups (Fig. 8). Such

clustering of sites into colony groups allowed genetic analyses to be performed within and among sites but also within and among colony groups that may better represent populations across the landscape of Camp Shelby. Additionally, such grouping allowed intensive landscape feature data to be collected between colony groups to assess the potential role of the landscape in affecting distribution of genetic variation.

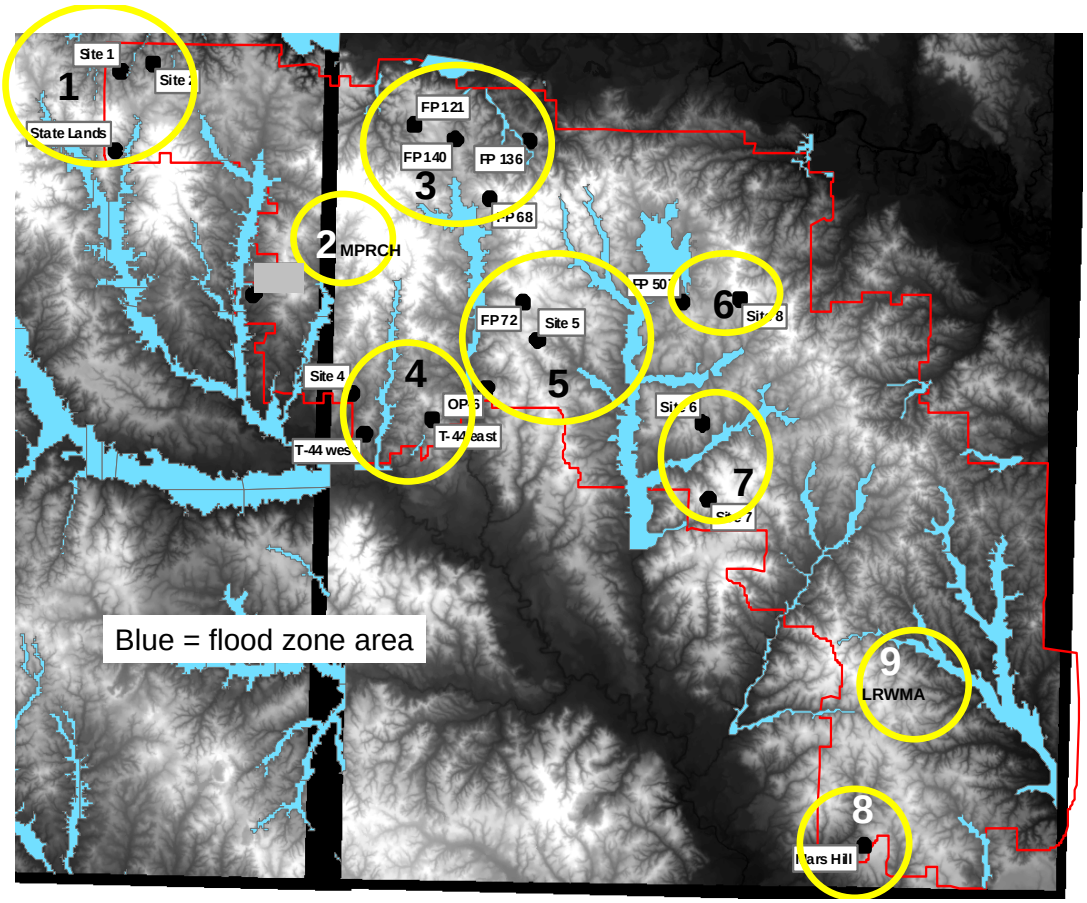

Figure 8. Clustering of tortoises into 9 functional "colony" groups over the landscape of Camp Shelby for conducting population genetic analyses.

In order to identify genetic variation in tortoises across the entire base and within colonies and also determine the relationship between genetic variation and habitat quality, population size, and sample size, several analyses of genetic variation were performed. Genetic variation was compared within and between study sites on Camp Shelby using allele richness and heterozygosity. Allele richness is the number of alleles present in a sample population. Heterozygosity is the proportion of individuals with two different alleles at a locus averaged across the nine loci. To evaluate differences among sites, only those sites that had at least 5 individual tortoises genotyped were included, which reduced the number of sites to 25. In addition to basic descriptive statistics, these genetic measures were used to evaluate the relationship between habitat quality and genetic variability using unpaired t-tests. We also tested for differences among the 6 treatments using ANOVA followed by Fisher's PLSD multiple

comparison method. Prior to statistical analyses, allelic richness was square root transformed and heterozygosity was arcsine-square root transformed. Because sites varied widely in the colony size (number of individuals/ colony group = 6-40), sample size effects were controlled by performing these analyses a second time using 6 randomly selected individuals per colony (for those sites with n > 6). Regression analyses were also performed to determine the relationship between population size and genetic variation and between sample size and genetic variation. Prior to regression analyses, population size and sample size were square root transformed. Analyses to test for relationships between genetic variation and population and sample size were also performed between colony groups as defined above. The relationship between genetic variation within each colony group and population size was evaluated as described for individual sites above.

Landscape population genetics

In order to identify genetic partitioning among populations (i.e., sites and colonies) across the base (i.e., population structuring) and how such partitioning relates to landscape variables, including geographic distance, landscape features, and other possible influential biological and environmental variables, the following analyses were performed. Differentiation among colonies or populations across Camp Shelby was tested using global tests not assuming HWE with 10,000 randomizations. Nei's (1973) F_{ST} was calculated to measure genetic structuring across the base and was evaluated statistically using bootstrap sampling over all loci to generate a 95% confidence intervals. F_{ST} is one measure of how genetic variability is distributed (within and between populations) across the landscape, and ranges from 0 to 1 where 0 = no genetic differentiation/population structure and 1 = complete genetic differentiation of populations. Wright (1965) described F_{ST} values and corresponding levels of differentiation as follows: 0-0.05 = little, 0.05-0.15 = moderate, 0.15 -0.25 = great, and > 0.25 = very great differentiation. Additionally, pair-wise F_{ST} values were used to describe genetic differentiation between each pair of populations. Following this calculation, the data were evaluated to test an isolation-by-distance model to determine if genetic similarity between populations could be explained solely by geographic distance between the populations. This pair-wise comparison between F_{ST} and geographic distance was evaluated using a Mantel test with 10,000 randomizations (Mantel 1967). Following these procedures, the landscape analysis described above was performed using colony groups as the sampling unit. In addition to these analyses, intervening landscape features were incorporated into the statistical analyses to further evaluate the impact of the landscape on the distribution of genetic variation. This procedure was performed by assessing the relationship between pairwise F_{ST} values and potentially influential landscape variables which were quantified as described below.

Landscape information over the aerial extent of Camp Shelby, including remote sensing-based data such as historical and recent aerial photographs, land cover, fire history, location of streams, other water bodies, roads, etc., were obtained from the natural resources group at Camp Shelby. Combining field-based data (such as vegetative types and cover - Fig. 9) and remote sensing information helps to better define and characterize a site and also provides addition information that could not be derived from field survey information alone. Landscape metrics, such as percentage of pine forest, length of roads, percentage of poor habitat, percentage of edge within a site, etc., were derived from the combined database. The remote sensing-based land cover data aids in providing a holistic characterization and evaluation of the entire environment of the

tortoise and identifies potential barriers to movements and to foraging and also availability of habitat corridors for movement. This information was used along with the tortoise health data to evaluate the relative importance of habitat quality, military activity, and various landscape features that could affect tortoise health and also to help explain the observed differences in tortoise health among sites and
treatments.

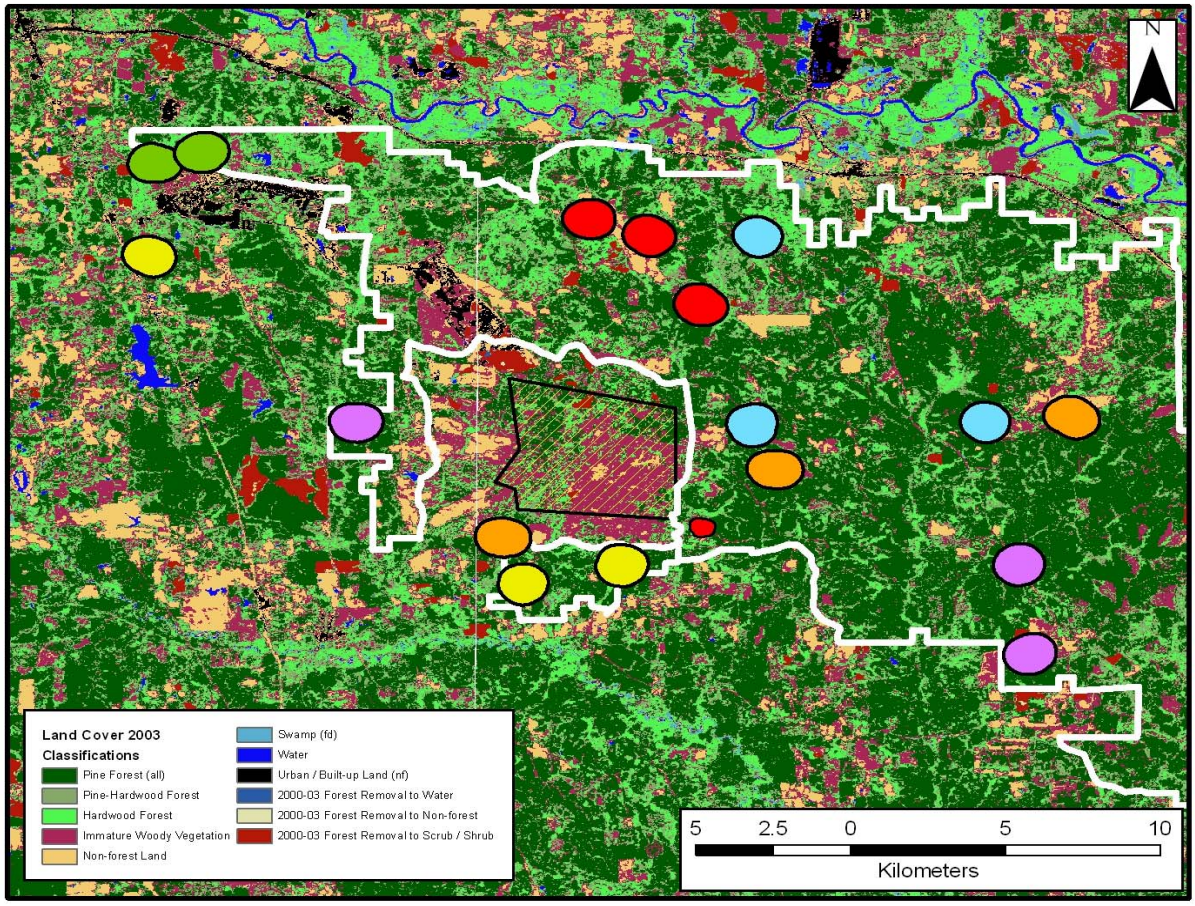

Figure 9. Landscape and major vegetative habitat features of Camp Shelby showing locations (color circles represent the 6 different treatments) of tortoise sample sites which were used in the population genetic analyses.

The types of landscape features existing between colonies or metapopulations of gopher tortoises at Camp Shelby could influence the movement and interactions among these colonies thus affecting their population genetics. For example, certain types of landscape features such as buildings and other physical barriers, water bodies such as streams, dense brush and vegetation, and lowland areas (swamps) would tend to impede or restrict movement of individuals between colonies while other features of the landscape such as open forest, grasslands, and roads would be favorable features that would encourage movement between colonies.

Landscape features were quantified within a 2 km wide corridor extending from the midpoints of each pair of groups by calculating the number of square km that each landscape feature comprised of the total area of the corridor **(**Fig. 10**).** 2 km-wide corridor is consistent with the average home range of gopher tortoises of 0.5-0.65 ha depending on gender and season (Heise and Epperson 2005). The favorable landscape features quantified in these corridors were longleaf pine forests, pine plantation/old field areas, maintained grassland, roads, and the number of tortoise burrows. The unfavorable (impediments to tortoise movement) landscape features quantified in these corridors were structures such as buildings, pine flatwoods, swamp and bottomland areas, and water bodies such as streams. Data for quantification of these landscape features were available from different types of GIS layers including vegetative cover, streams, and roads and these layers were obtained from the Camp Shelby Natural Resources group. Information on the location of active and inactive tortoise burrows was available in data spreadsheets and then plotted on the appropriate Geographic Information System (GIS) layer. ArcCatalog software (ESRI, Inc.) was used for data management and ArcMap software (ESRI, Inc.) for displaying and querying data.

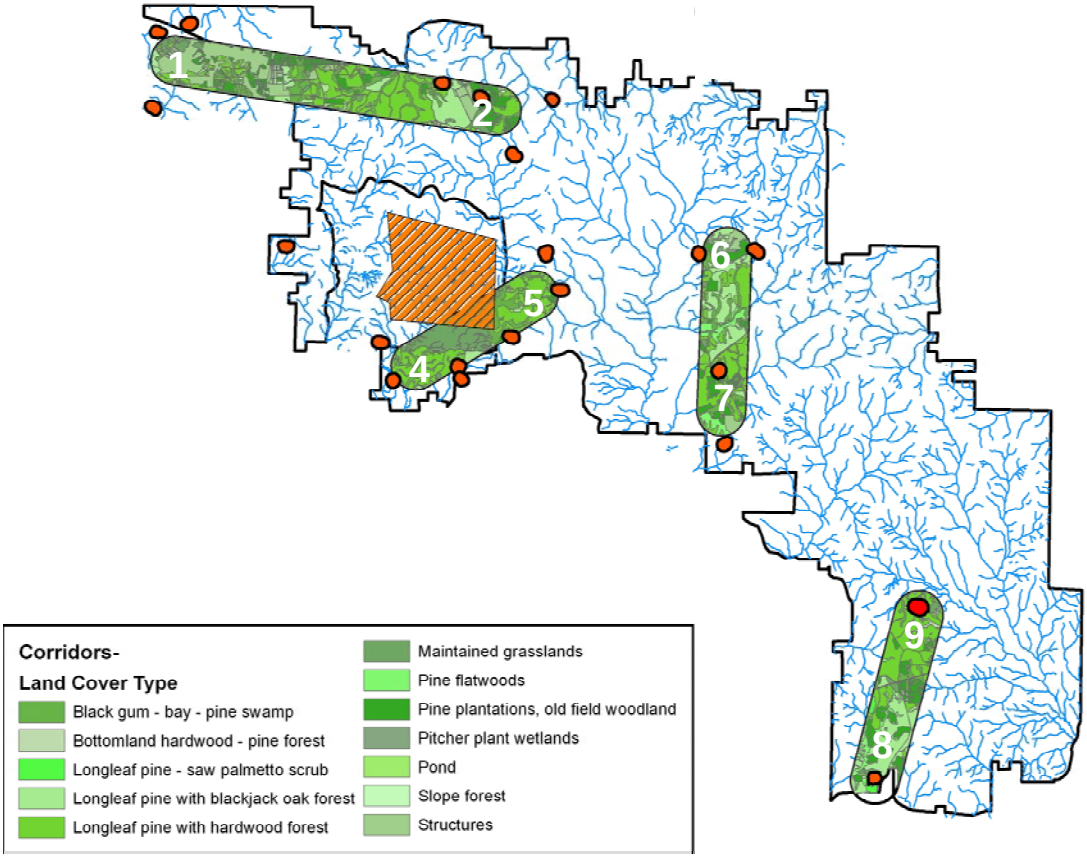

Figure 10. Examples of the 2 km- wide movement corridors for tortoises between pairs of colony groups on Camp Shelby.

Reproductive studies
In addition to the reproductive studies conducted as a component of the tortoise health investigations in 2006, special reproductive studies were also conducted in the spring-summer of 2007 and 2008 that focused on specific areas of the base and aspects of tortoise reproductive health that were identified in the 2006 investigations to be of particular concern. These reproductive studies involved a selected suite of sites that represent four different treatment effects including (1) good habitat and no military activity, (2) good habitat and high military activity, (3) poor habitat and no activity, and (4) good forested habitat and no activity. In May/June of 2007 and 2008, tortoise nests were identified by daily burrow apron inspection at pre-selected sites. When a nest was located, the eggs were counted and then re-buried (with a nest protector) for a period of 60 ± 5 days. Following this period of burial, eggs were dug up, transported back to the field office, weighed, and measured. Each clutch of eggs was placed into a bin with a 1:1 ratio of vermiculite and distilled water (by weight), covered with plastic wrap, and placed in a constant-temperature incubator for the remainder of the incubation period.

The reproductive-related parameters that were measured included fecundity (clutch size), egg quality (egg size), and hatching success. These parameters were then compared by treatment, by site, by habitat quality, and by military activity. Hatching success data from 2006 were not used due to inconsistent incubation methods from that year. To check for differences based on habitat quality, the poor quality sites were combined (T4 and T5) and the grassland sites were combined (T1, T2, and T3). The good quality forested sites (T6) were analyzed as a separate treatment. To check for differences based on military activity, the two treatments thought to have the highest military effects were combined (T2 and T3), and compared to the combination of the other four treatments (T1, T4, T5, and T6). Differences were considered significant at $\alpha \leq 0.05$. If a statistically significant difference was found, pairwise comparisons were performed (when applicable) to determine which particular treatments had the greatest differences.

Statistical analysis
Individual bioindicator analysis

Body condition Index - The body condition index (BCI) of each tortoise was measured by calculating the residuals from a linear regression plotting body weight or mass (kg) as the dependent variable against straight carapace length (mm) as the independent variable. A constant, 0.77, was added to every BCI to ensure that all points were positive. Data were log transformed for analysis and three outliers, calculated using Grubb's Test for Outliers, were removed. The BCI calculation for each treatment was based on combining males and females because the ANOVA indicated that there was no effect of gender on the BCI (P=0.99). A one-way analysis of variance (ANOVA) was used to test for significance differences in the BCI among the six treatment groups.

Stress hormones and immune system response - Baseline cortisol levels (stress hormones) were log transformed prior to analysis to ensure normality and heterogeneity of variance. Eight tortoises from the total collected were determined to be outliers according to Grubb's Test for Outliers and were excluded from the analysis. For comparison of cortisol levels among treatments, males and females were combined in the analysis because the ANOVA indicated that there was no effect of gender on these levels (P=0.50). For the bacterial killing assay, a one-way

ANOVA was used to test for global differences among treatment groups and then a Tukey-Kramer post hoc analysis was used to determine which specific pairs of treatments were significantly different.

Oxidative stress, DNA damage, blood chemistry, hematology and reproductive condition - A one-way ANOVA was used to determine statistical significance among treatments for the oxidative stress, DNA damage, blood chemistry, hematology, and reproductive parameters with significance set at $P<0.05$. Tests for homogeneity of variance for individual response variables among treatments were conducted using Bartlett's test for equal covariance matrices (Johnson and Wichern 1992).

Multivariate-integrated bioindicator analysis
A canonical discriminant analysis procedure was used to assess the integrated health status of tortoises among the six experimental treatments at Camp Shelby. To examine the integrated responses of tortoises for each experimental treatment, the individual bioindicator variables were considered jointly within a multivariate context using this canonical discriminant analysis procedure (Adams et al. 1994). Canonical discriminant analysis generates new sets of variables that are linear combinations of the original bioindicators which account for most of the variation among treatment groups. This procedure provides a reduced set of canonical variables that are the most important and influential for differentiating responses among treatment groups. The greatest difference (or the highest discriminatory ability) among treatment groups is typically represented by the first canonical variate, the next greatest discriminatory ability by the second canonical variate, etc. This procedure also identifies the canonical variate means that are most closely related among treatment groups. The 95% confidence regions (radii) were used to indicate the uncertainty associated with the estimated mean canonical variates from each treatment group. The center of each of the confidence regions is the mean value of the canonical variables. A stepwise variable selection procedure was then used to identify those bioindicator variables which were the most important or influential in causing differentiation or separation among treatment groups.

Gopher Frogs-Eglin AFB

Experimental field design

The sampling design for gopher frogs at Eglin AFB is based on three main treatment categories which include relatively high disturbance areas, medium to low disturbance areas, and no disturbance or reference areas (Fig. 11). Based on our initial field surveys, high disturbance areas are characterized by relatively high levels of habitat modification and also relatively high levels of explosive residuals in the soil surrounding the candidate sample ponds and/or in the water of sample ponds. Low or medium disturbed areas are characterized by either some level of explosive residuals above background values or at least some moderate degree of habitat modification. Other variables included in the ranking of sites according to level of military activity are amount of ordnance fragments/unit area, amount of cratering/unit area, amount of soil and habitat disturbance due to tracked vehicles or other heavy equipment, amount of burning or scorching at a site, and level of vegetation control resulting from application of herbicides and/or roller chopping practices (Table 2). Reference areas were chosen based on their relatively

undisturbed state. Each of these three types of treatments includes three replicate sites and each replicate consist of 2-4 individual sample ponds or wetlands. Specific sites represented by the high disturbance treatment are ponds or wetlands within the C-52 N and "cat's eye" area of the C-52 bombing range, two ponds on the far western end of the B-70 bombing range, and ponds within the C-74 range where test firing of rockets occurs (Fig. 11). Most of these high disturbance areas are characterized by relatively high levels of habitat modification as evidenced by numerous bomb craters, sparse vegetation, abundant metal fragments from exploded ordnance, and detectable levels of explosive residuals such as RDX, HMX or TNT, either in the soil surrounding the sample ponds, in the pond water itself, or in both the soil and water. Medium or low disturbance areas are represented by the lower end of the C-52 range (C-52 C and C-52A), and one pond on the lower western end of the B-70 range (Fig 11). A series of reference ponds are located west of the C-74 range and also south of the B-70 range (Fig. 11). The aquatic habitat types represented within this experimental field design vary from rather large shallow water ponds such as Bull pond (5-10 acres) on the B-70 range and a series of reference ponds (2-3 acres) south of the B-70 range, to smaller wetland seeps and larger bomb craters containing water and vegetation along Bay Branch. Bay Branch is located immediately west of the "cats eye" on the C52N range.

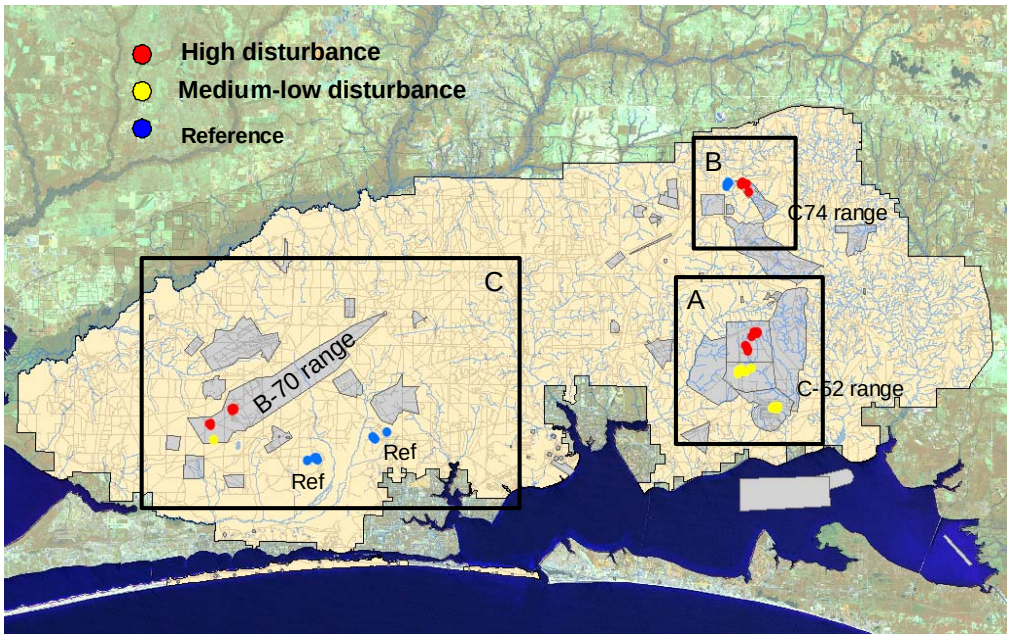

Figure 11. Sampling design and site locations for collection of gopher frogs at Eglin AFB. Sites include high disturbance areas on active ranges (red), medium-low disturbance (yellow), and reference areas (blue).

Table 2. Assessment of military activity at representative sample sites at Eglin AFB. Military activity was assessed by determining the amount of explosive residuals, ordnance, cratering, soil disturbance, and burning at each site.

Sample site	Explosive residuals	Ordnance/ unit area	Cratering/ unit area	Soil disturb.	Burning/ scorching	Composite score	Military activity
C52N-1	3	3	3	3	2	14	High
C52N-2	3	3	3	3	1	13	High
C52N-3	3	3	3	2	2	13	High
C52C-1	1	1	2	2	1	7	Moderate
C52C-2	1	2	2	2	1	8	Moderate
C52C-3	1	1	2	2	2	8	Moderate
Reference-1	0	0	0	1	2	3	Low
Reference-2	0	0	0	1	1	2	Low
Reference-3	0	0	0	0	2	2	Low

Criteria for assessing military disturbance

3 = moderate high
2 = low to moderate
1 = none to low

At each site or sample location several collection traps were placed separately or along 30m drift fences. Collection traps for frogs were modified by installing styrofoam inside for flotation. The traps and their associated drift fences were installed at each of the 40 sample sites which represent (1) three major experimental treatments (high, medium, and low disturbance) including the reference treatment, (2) three replicated areas for each treatment type, and (3) from 2-5 separate ponds or wetland areas at each replicated area. Each 30-m drift fence was held in place with six evenly-spaced 6-ft metal stakes. Four frog traps were associated with each 30-m fence, two at each end of the fence on opposite sides (Fig. 12). Traps were rigged with metal rods and O-rings to float up and down with the rising and falling of water in the sample ponds. At the 40 sample sites at Eglin we installed 162 x 30m drift fences representing a total of 5700 feet. The total number of traps deployed at all sites was 940 including those associated with the drift fences and single traps which were also placed at each sample site. The number of traps deployed at each pond or wetland area was standardized based on the perimeter and total surface area of each wetland area. Values for pond surface areas and perimeter were obtained by GPS readings. The number of traps was standardized at each sample site for the purpose of estimating relative population abundance of frogs among sites. Representative habitats and sites where cages and drift fences were placed over Eglin AFB are shown in Figures 13-15.

Sampling devices deployed at Eglin

- Total sites with drift fences and traps - 40
- Total number of 30m drift fences with cages- 162
- Total feet of drift fencing- 5700
- Total number of traps at 40 sites- 940
- Number of traps at each site standardized based on pond surface area

Figure 12. Collection devices including drift fences and traps for sampling of gopher frogs at 40 sites representing 3 major treatments types at Eglin AFB.

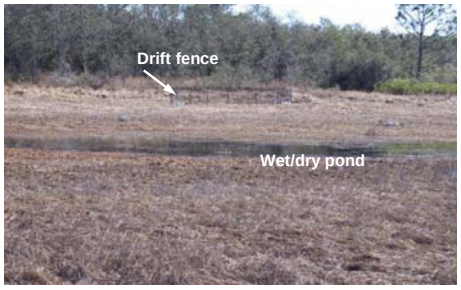

Figure 13. Examples of drift fences and frog traps placed in different types of habitats including a wet/dry pond area (upper left), a seep area (upper right), an old bomb crater (lower left), and a new bomb crater.

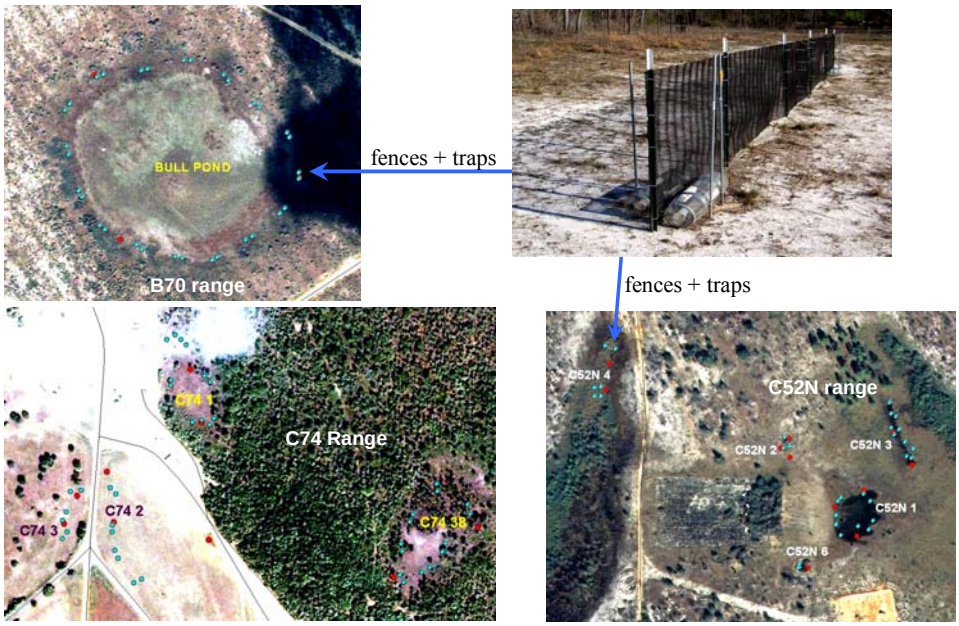

Figure 14. Examples of the high disturbance sample sites (B70 range, C74 range, C52N range) for collection of gopher frogs at Eglin AFB. Each blue dot at these sites represents a 30m drift fence and its associated frog collection traps (upper right photo).

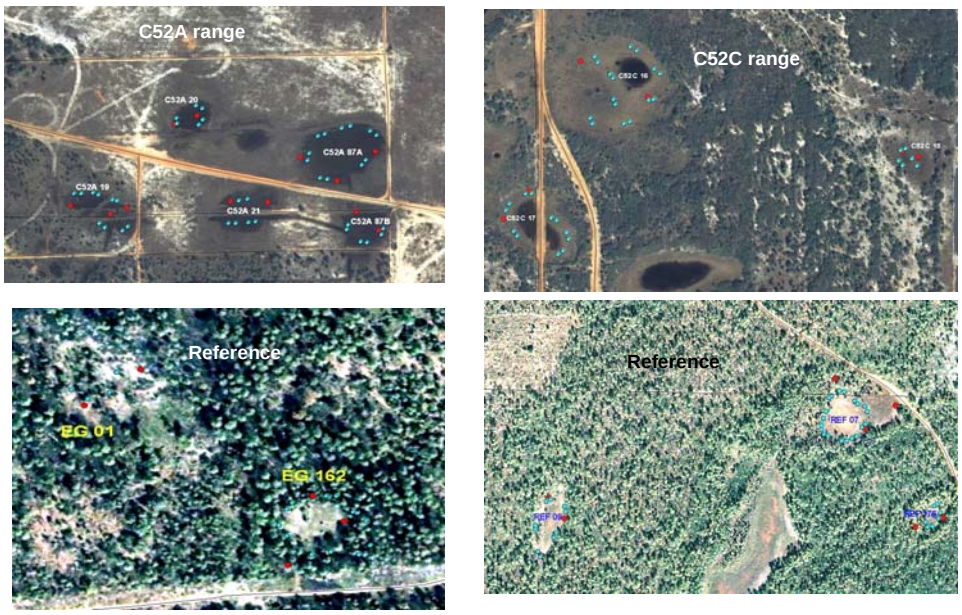

Figure 15. Examples of the low disturbance sample sites (C52A & C52C ranges) and reference sites for collection of gopher frogs at Eglin AFB. Each blue dot at these sites represents a 30m drift fence with frog collection traps (upper right).

Aerial images of representative sample sites for each treatment type include a low disturbance area on the C52A range (Fig. 16a), a reference area (Fig. 16b), a high disturbance area on the C74 test missile range (Fig. 16c), and a high disturbance area on the C52N range (Fig. 16d). The home range of the gopher frog around each sample pond is represented by a yellow border which is approximately 300m (Richter et al. 2001). The chemical and habitat studies were designed, therefore, to thoroughly characterize the environment within the 300 m home range of the gopher frog where it resides during most of its life history.

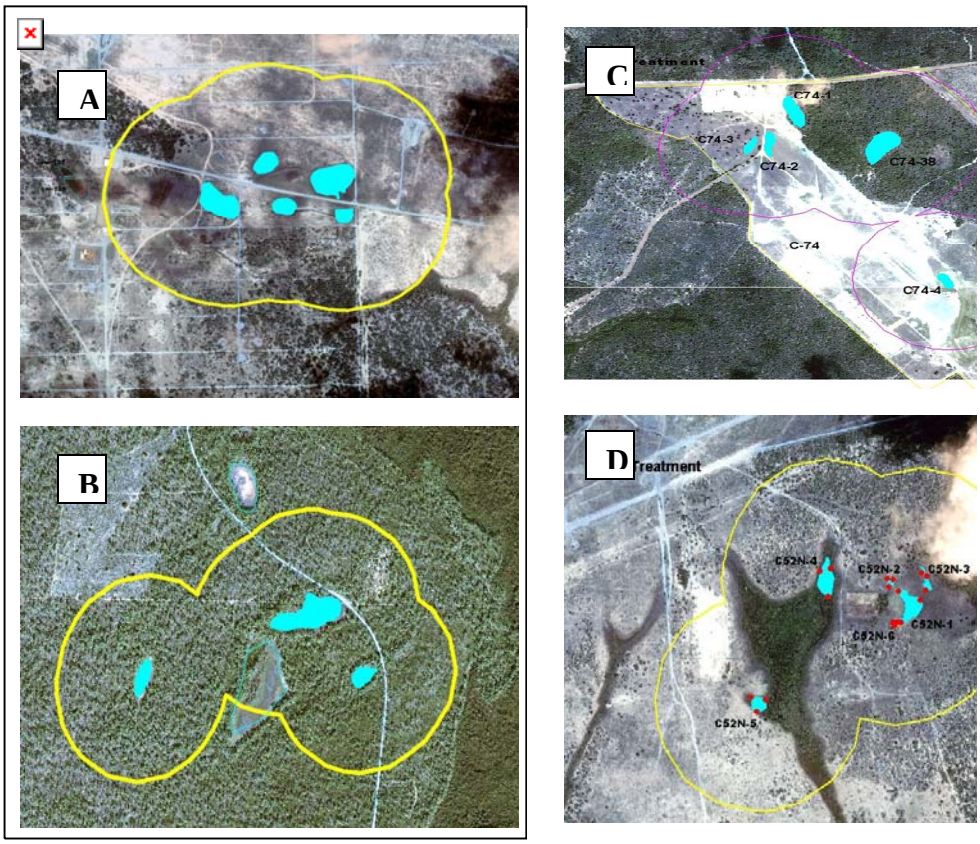

Figure 16. Aerial images of representative experimental treatment types at Eglin AFB for sampling gopher frogs including a low activity site (C52 range) (A), a reference area (B), a high activity site (the C74 range) (C), and another high activity site (the C52N range) (D). Yellow borders are the 300m home range boundaries (radii) of the gopher frog. Detailed chemical and habitat characterization studies were conducted within these boundaries.

Habitat characterization

Habitat characterization studies were also conducted at Eglin AFB in conjunction with the chemical characterization (explosive residual) sampling. For each pond or wetland area, characteristics of pond morphology, pond water quality, pond substrate, vegetation, and military disturbance were measured (Fig. 17). Pond metrics ranged from the highly quantitative (e.g., pond length/width/depth, water temperate, pH, dissolved oxygen, conductivity), to semi-quantitative (e.g., % plant cover, disturbance rankings), to qualitative descriptions (e.g., plant

community descriptions, forest stand age, site sketch maps, etc.). In addition, at each pond GPS coordinates and digital photos were obtained.

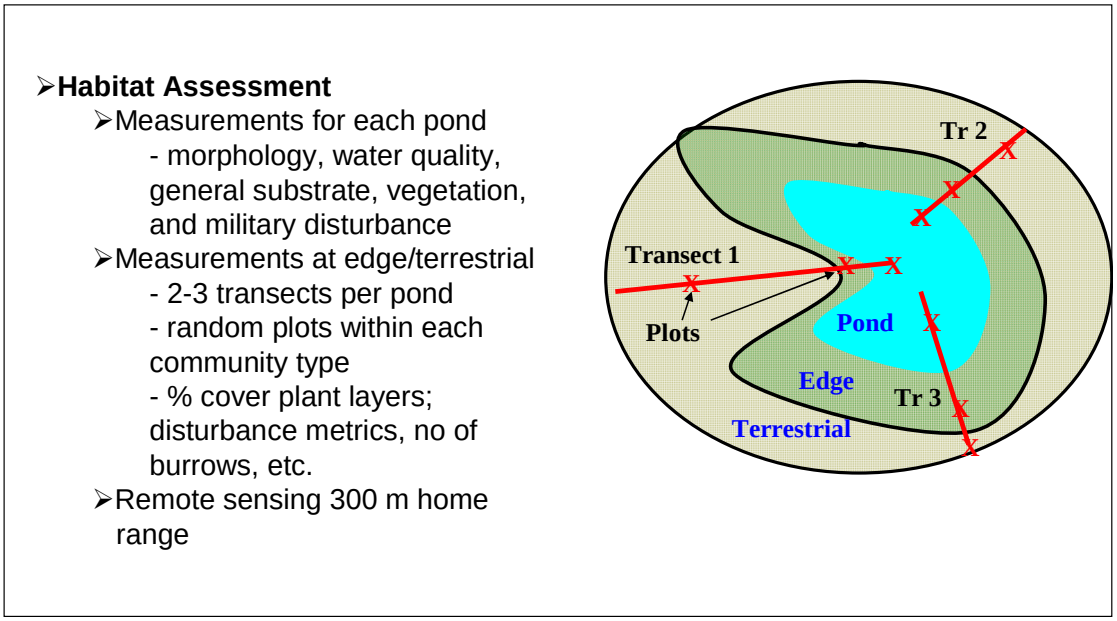

Figure 17. Basic procedures for characterizing the habitat of gopher frogs at Eglin AFB. To thoroughly characterize the environment of the gopher frog, various basic habitat and habitat disturbance metrics were measured along transects which include plots in the pond environment where frogs breed, the aquatic-terrestrial edge environment, and the terrestrial environment where frogs reside during most of their life history.

To provide a means for comparing habitat attributes between ponds and treatments, two to three random transects were established at each pond (Fig. 17). Plant cover was obtained within 1 meter quadrants placed randomly in each plant community type along each transect line. Typically, each pond included aquatic habitat (defined as open water, but potentially dominated by submerged or floating plants species), a shore/edge community with emergent plants and/or hydrophilic species, and terrestrial habitat (in most cases outside the range of pond flooding). Summary determinations of plant layers (percent total herbs, submerged or floating plants, small and large shrubs, vines, etc.), ground characteristics of importance to frogs (e.g., general soil type, percent bare ground, percent litter, number of burrows, etc.), disturbance metrics (amount of ordnance, cratering, etc.), and canopy cover and basal areas were determined for each quadrant. Large areas of terrestrial habitat between ponds were assessed primarily by using remote sensing techniques in combination with ground-truthing information obtained from the terrestrial plots.

Chemical characterization

Chemical characterization studies were conducted at each of the experimental field sites at Eglin AFB according to the methods of Jenkins et al. (2005) to determine the levels of explosive residuals in soil and water (Fig. 7). Working with Tom Jenkins and Alan Hewitt from ERDC/CRREL, soil samples were collected from each experimental site with a coring tool and the top 2cm of material (soil and vegetation) retained. At each site 2-3 sampling grids of 2500m^2 were laid out and within each 50 x 50m^2 grid, approximately 100 surface core samples were collected at evenly spaced intervals. Thus, levels of explosive residuals in the soil for each treatment replicate were based on 200-300 increments or subsamples which were composited from each grid for analytical analysis.

RESULTS

Most of this section reports on the results of the gopher tortoise studies at Camp Shelby since only the chemical and habitat characterization studies were conducted at Eglin AFB due to the extensive drought and the inability to capture frogs for the biological component of this project.

Gopher Tortoises-Camp Shelby

Individual bioindicator analysis

A variety of biological responses including the sensitive and rapidly-responding biomarkers and the more integrative and ecologically-relevant bioindicators were measured on tortoises collected at each sample site. The relationship between biomarkers or responses measured at the lower levels of biological organization (biomolecular, biochemical, etc) and the more ecologically-relevant bioindicators of effects are shown in Figs. 1 and 18. These measures represent a gradient in biological responses from early warning and sensitive indicators to more ecologically-relevant but less sensitive indicators.

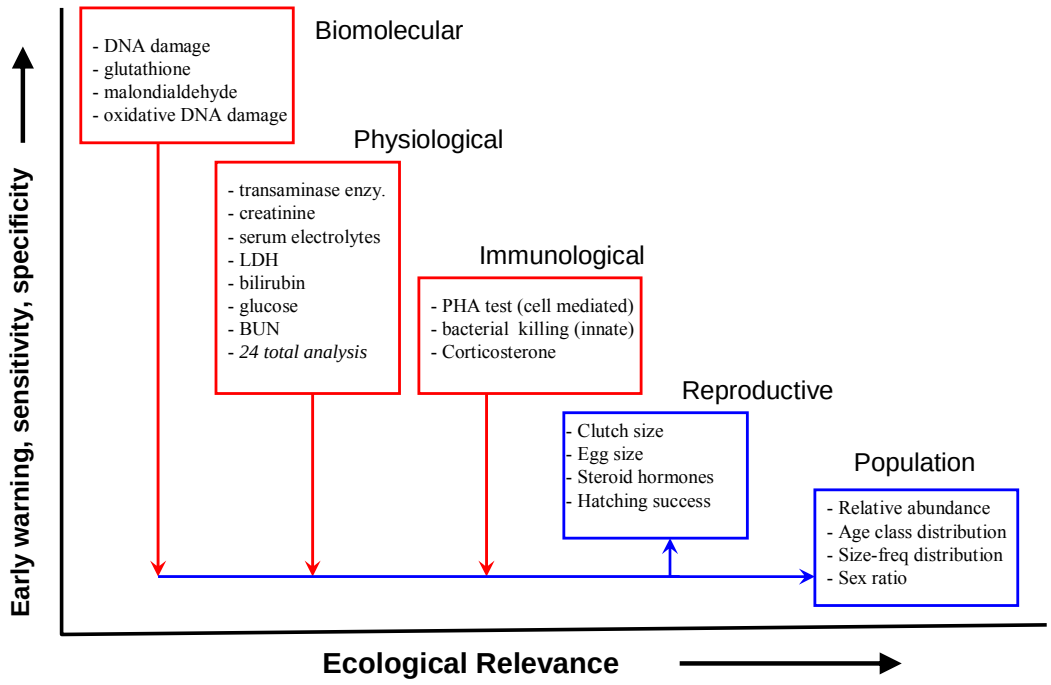

Figure 18. Biomarkers and bioindicators measured in gopher tortoises sampled from 20 sites at Camp Shelby. These measures represent a gradient in biological responses from early warning and sensitive biomarkers (red boxes) to more ecologically-relevant but less sensitive bioindicators (blue boxes).

Data analysis for individual bioindicator responses related to the evaluation of tortoise health and condition among treatments (six different treatments involving different combinations and levels of military activity and habitat) were based on the following functional response groups (1) carbohydrate/protein metabolism, (2) oxidative stress, (3) organ dysfunction, (4) electrolyte homeostasis, (5) stress hormones, (6) body condition, (7) immune system competence, (7) hematological response, and (8) reproduction condition or competence.

Carbohydrate/protein metabolism - Serum glucose and protein levels indicate not only the ability of an organism to maintain steady state regulation and metabolism of carbohydrates and proteins under environmental stress conditions, but also reflect, to some extent, quality of the diet. Serum glucose for tortoises varied from low levels at the good habitat-no activity treatment (hereafter designated as Treatment 1 = T1) and the poor habitat-no activity treatment (hereafter designated as Treatment 5 = T5) to high levels at the good habitat-low activity treatment (hereafter designated as Treatment 3 = T3) and the open forest-no activity treatment (hereafter designated at Treatment 6 = T6) (Fig. 19). Intermediate glucose values occurred for tortoises at the good habitat-high activity treatment (hereafter designated at Treatment 2 = T2) and the poor habitat-low activity treatment (hereafter designated as Treatment 4 = T4). Only T3 and T6 were significantly higher than the reference, T1. Total serum protein varied from low values at T4 and T5 to highs at T2, T3 and T6, with these three treatments being significantly higher than the other treatments (Fig. 19). Interestingly, patterns of high and low values of glucose and total protein among treatments generally reflected each other, with low glucose values among treatments generally indicating lower protein levels and the higher glucose and protein levels generally varying together.

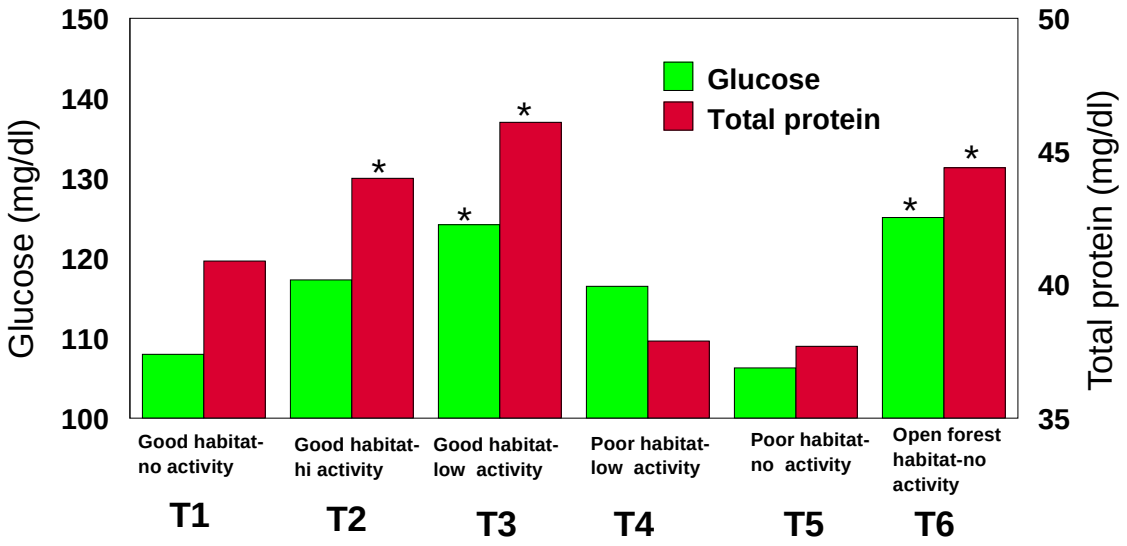

Figure 19. Indicators of carbohydrate-protein metabolism in gopher tortoises at the six experimental treatments (T1-T6). Asterisks above histograms indicate that values are significantly different (P<0.05) compared to the reference T1.

Oxidative stress enzymes - Glutathione, an oxidative stress enzyme, is an indicator of the level of oxidative stress in tissues. More specifically, as a measure of oxidative stress to cell membranes, malondialdehyde is an indicator of the amount of lipid peroxidation (caused by oxyradicals) occurring at the cell membrane level which can cause damage to cell proteins and DNA. Levels of malondialdehyde, an indicator of lipid peroxidation, were lowest at T1, T2, and T5 and significantly higher than the reference (T1) at T4 and T6 (Fig. 20). In contrast to this pattern, gluthathione, a more general indicator of oxidative stress in organisms, was lowest at T6, highest at T2, and displayed intermediate values at the remaining treatments (Fig. 20). For three of the six treatments, high levels of gluthathione were generally associated with very low values for lipid peroxidation.

Organ dysfunction - Several serum chemistry parameters serve as indicators of damage or injury to organs such as liver, kidney, and spleen. Aspartate aminotransferase (AST) is a specific indicator of liver damage while lactate dehydrogenase (LDH) is a more generalized indicator of cell and tissue damage. AST was lowest at T1, T4, and T5 while levels in tortoises from T2, T3, and T6 were significantly elevated above the reference (T1) (Fig. 21). Levels of LDH were somewhat similar among treatments except for T2 and T3 which were significantly elevated above the reference (T1) (Fig. 21). It is interesting to note that the two treatments characterized by both significantly elevated AST and LDH levels were the two treatments that were characterized by some level of military activity (T2 and T3).

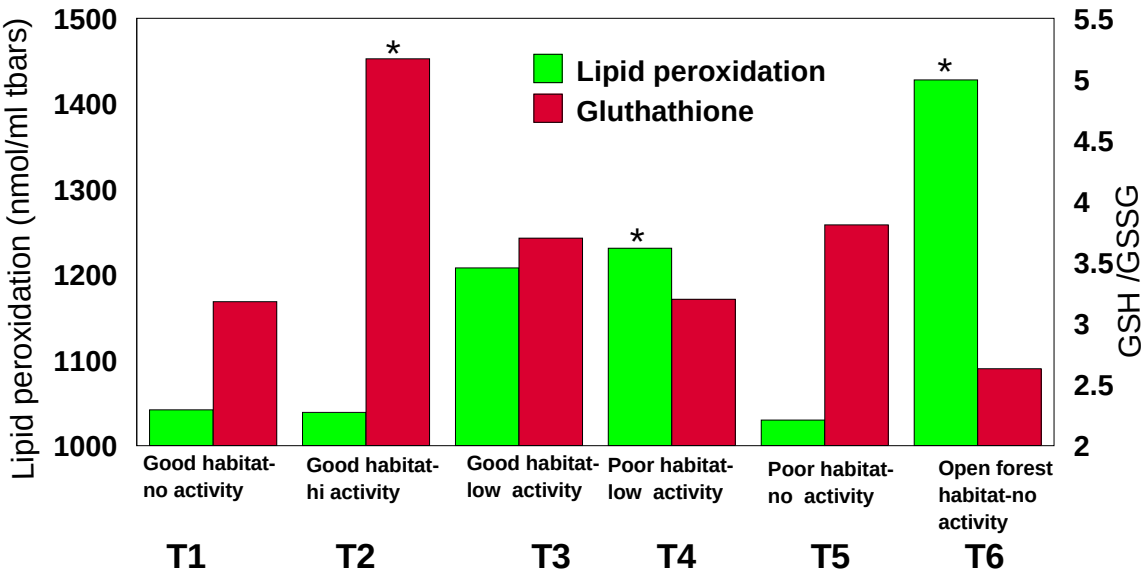

Figure 20. Indicators of oxidative stress in gopher tortoises at the six experimental treatments (T1-T6). Asterisks above histograms indicate that values are significantly different (P<0.05) compared to the reference T1.

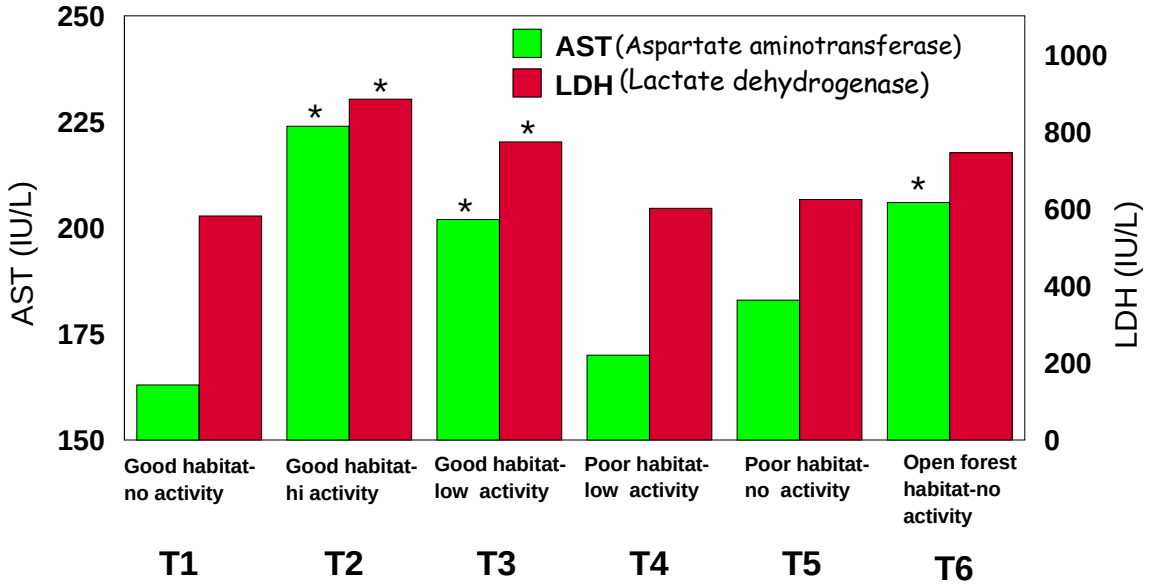

Figure 21. Organ dysfunction indicators in gopher tortoises at the six experimental treatments (T1-T6). Asterisks above histograms indicate that values are significantly different (P<0.05) compared to the reference T1 (good habitat-no activity).

Electrolyte homeostasis - Levels of serum electrolytes such as calcium, magnesium, and phosphorus have several important physiological functions such as maintenance of proper osmotic homeostasis, reproductive integrity, and as a blood buffering mechanism. At the two poor habitat treatments (T4 and T5) levels of Ca, Mg, and P were significantly lower than that for tortoises collected from the three good habitat treatments (T1-T3) while only Mg was significantly lower at T6 compared to the good habitat treatments. Levels of Ca, Mg, and P were very similar among treatments T1-T3, the good habitat treatments (Fig. 22).

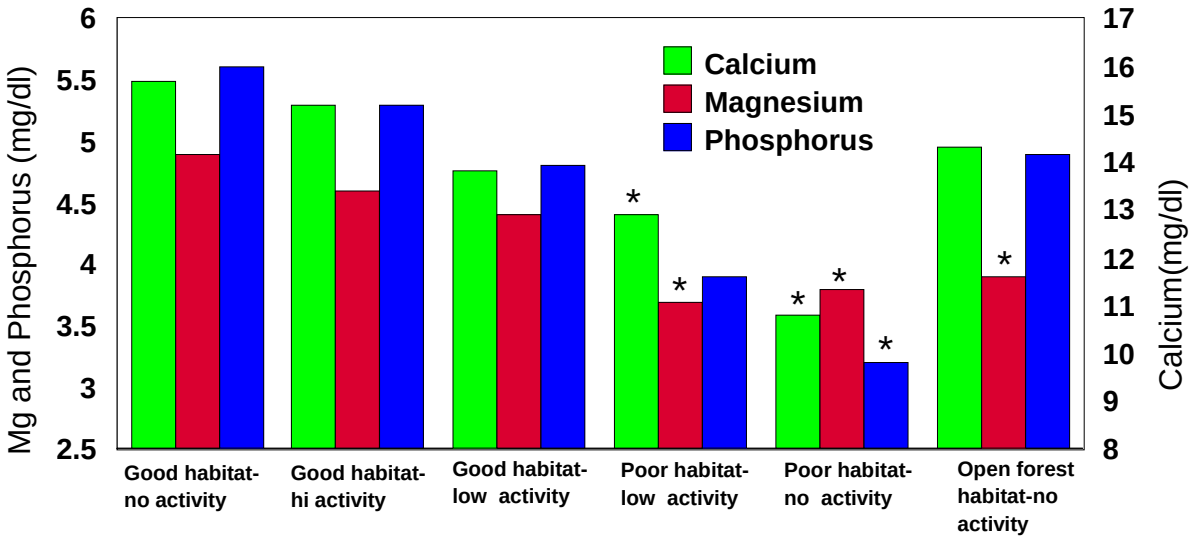

Figure 22. Indicators of electrolyte homeostasis in gopher tortoises at the six experimental treatments (T1-T6). Asterisks above histograms indicate that values are significantly different (P<0.05) compared to the reference T1.

Stress hormone - Cortisol is a generalized stress hormone that reflects an organism's stress response to a variety of factors in the environment. There was a significant difference in cortisol levels among some of the treatment groups (P<0.001) with levels being similar at T1, T4, and T5 and significantly elevated in tortoises from T2, T3, and T6 compared to T1 (Fig. 23). Tortoises at T6 had the highest cortisol levels and those individuals from T1 (the reference) had the lowest levels. Tortoises from T1 that had the lowest cortisol levels had the highest body condition index (BCI) and tortoises from T6 where cortisol was the highest had the lowest BCI (Fig. 23). Thus it appears that body condition or overall health of tortoises, as represented by the BCI, may be related in some way to the level of stress in these individuals as illustrated by the baseline cortisol levels.

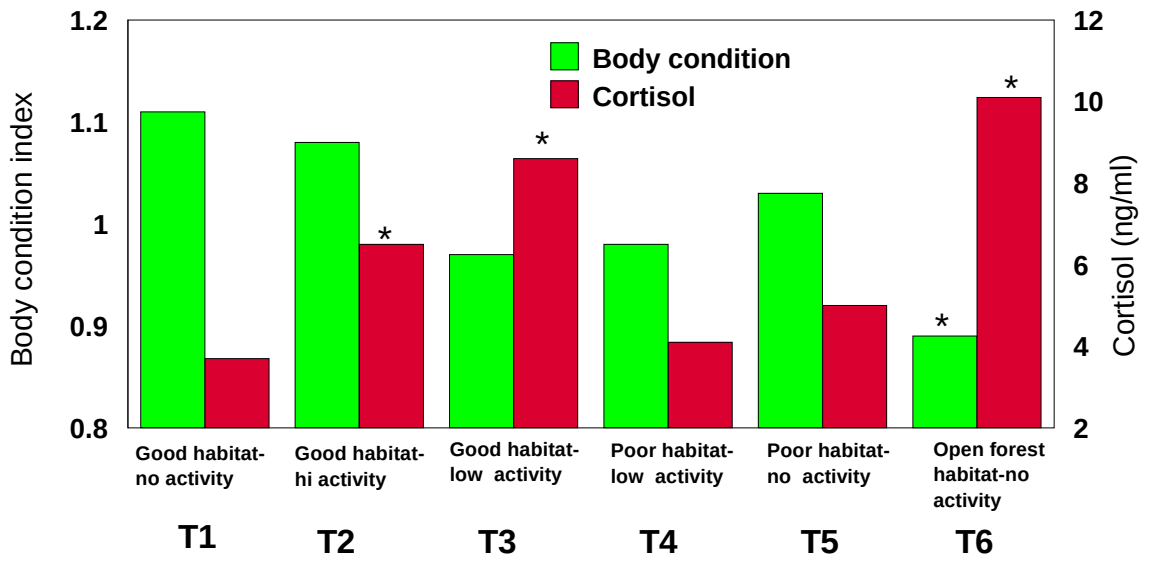

Figure 23. Stress hormone and body condition indicators in gopher tortoises at the six experimental treatments (T1-T6). Asterisks above histograms indicate that values are significantly different (P<0.05) compared to the reference T1 (good habitat-no activity).

Body condition - The overall health or condition of tortoises is represented by the body condition index (BCI) which reflects the integrated response of all environmental factors impinging on the tortoise in its immediate environment at Camp Shelby. Even though the BCI was higher at T1 and T2 compared to the other treatments, only at T6 was the BCI significantly lower compared to the main reference treatment (T1) (Fig. 23). Habitat considerations alone appears to have little effect on the BCI because the two poor habitat treatments, T4 and T5, had intermediate BCI values compared to the good habitat treatments (T1-T3)

Immunological response- the bacterial killing assay - Only tortoises from T2, the treatment with the highest military activity, had significantly lower bacterial killing ability than the other five treatments (Fig. 24). Even though T6 had the highest bacterial killing ability, it was only significantly higher than T2. As indicated by this assay, the immune system competence of tortoises may be compromised at T2, the treatment with the highest level of military activity. Based on this index alone, it appears that habitat quality has less affect on immune system competence than does level of military activity.

Hematological response - Lymphocytes and heterophils (or neutrophils in mammals) typically compose the highest percentage of leucocytes in the blood. The primary function of lymphocytes is to produce specific antibodies which provide defenses against pathogenic micro-organisms such as viruses, bacteria, and fungi. The primary function of heterophils is to phagocytize, or engulf, foreign particles particularly in infected tissue.

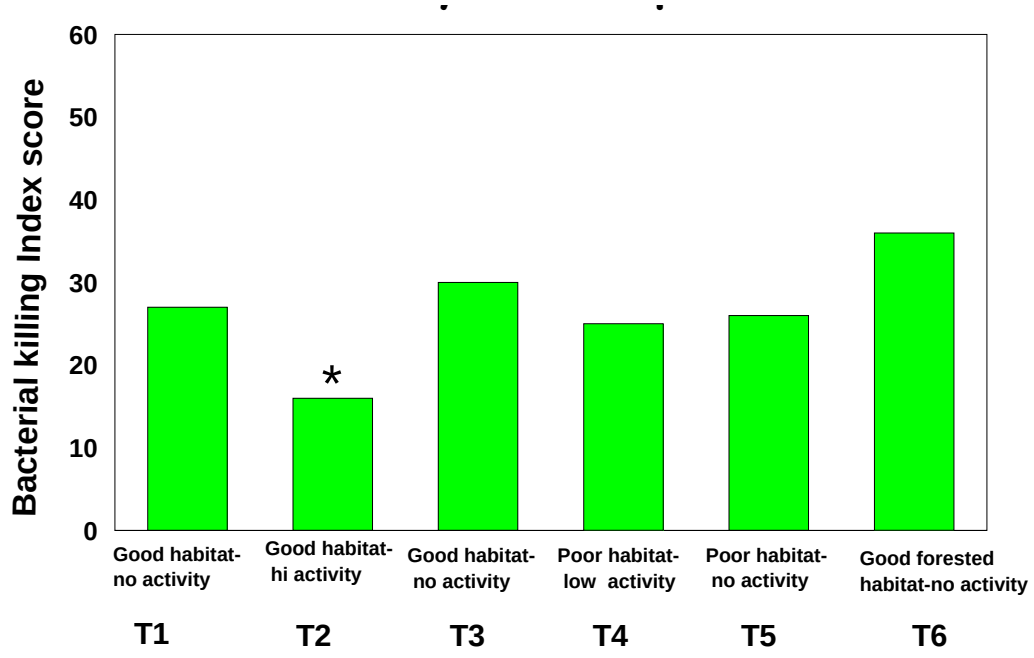

Figure 24. Immune system status as indicated by the results of the bacterial killing assay in gopher tortoises at the six experimental treatments (T1-T6). Asterisks above histograms indicate that values are significantly different (P<0.05) compared to the reference T1 (good habitat-no activity).

Lymphocytes were significantly lower for tortoises from T2 and T6 compared to the reference, T1 and also compared to T5 (Fig. 25). In contrast, these two treatments (T2 & T6) had significantly higher heterophils, or the ability to engulf foreign invaders in the blood, compared to T1 and also compared to T5. Also, heterophils were significantly higher at T2-T4 compared to T1 and T5.

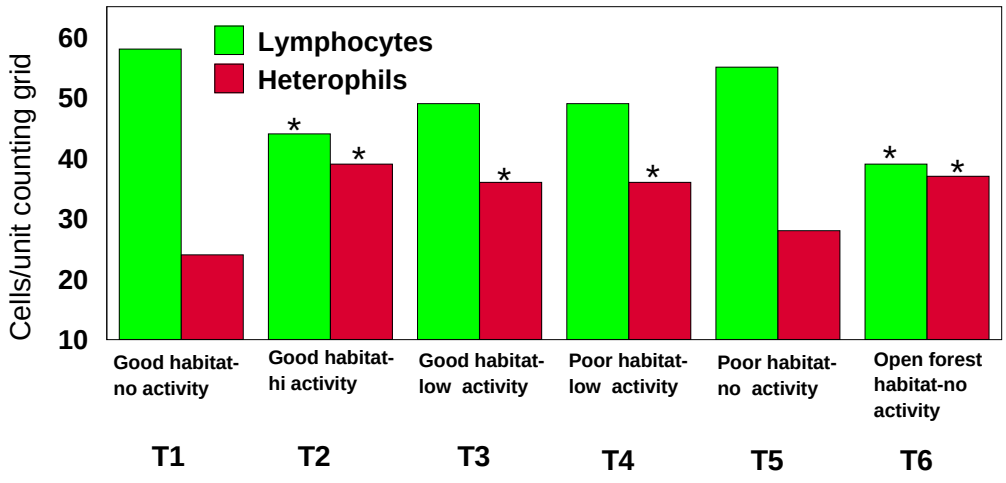

Figure 25. Indicators of hematological status in tortoises at the six experimental treatments (T1-T6). Asterisks above histograms indicate that values are significantly different (P<0.05) compared to the reference T1.

Upper Respiratory Tract Disease -Tortoises from all sample sites were analyzed for the presence of *Mycoplasma agassizii,* the bacterium responsible for upper respiratory tract disease (URTD) in tortoises. Tortoises with an antibody titer of less than 32 were designated as being negative for the presence of the bacterium, those tortoises with a titer of 32-64 were suspect carriers for the disease, and tortoises with a titer of greater than 64 were positive for the bacterium. Only one tortoise from T6 (good forested habitat-no military activity) tested positive for the presence of *Mycoplasma agassizii* antibodies and 7 tortoises were suspect for the disease (3 from T1, and one each from T2, T3, T4, and T6). These results indicate that there is no apparent pattern in the incidence of this disease as a function of habitat type or level of military activity. In fact, of those 8 tortoises that were either suspect or positive for URTD, 5 were captured on sites with no military activity (T1 and T6), 5 were sampled from ruderal (grass) habitats, and 3 were from forested areas. Previous studies of URTD at Camp Shelby by Epperson (2005) tested 124 tortoises from 15 sites and no tortoises tested positive but 17 individuals were considered suspect. Of these 17, only two individuals were associated with heavy military training areas.

Reproductive status

Assessment of reproductive status for the three years of this study included the main sample year (2006) and the special studies conducted for reproductive fitness during 2007 and 2008. Parameters measured related to reproductive status in 2006 included clutch size and egg size and parameters measured in 2007 and 2008 were clutch size, egg size, egg hatching success, and nest hatching success. Average clutch sizes and egg sizes for the six treatments over the three years (2006-2008) are shown in Fig. 26. For hatching success in 2007 and 2008, two different averages were calculated: overall nest success (either 0% or 100%, based on the successful hatching of at least one tortoise), and individual egg hatching success. These average values were calculated for the combined 2007-2008 nesting seasons (Fig. 27). There were no statistically significant differences found in clutch size, egg size, or egg hatching success by treatment group, by site, by military activity, or by habitat quality. However, clutch size was found to have a significant linear relationship with gravid female carapace length ($r^2 = 0.88$, $P < 0.05$, $n = 20$; Fig. 28).

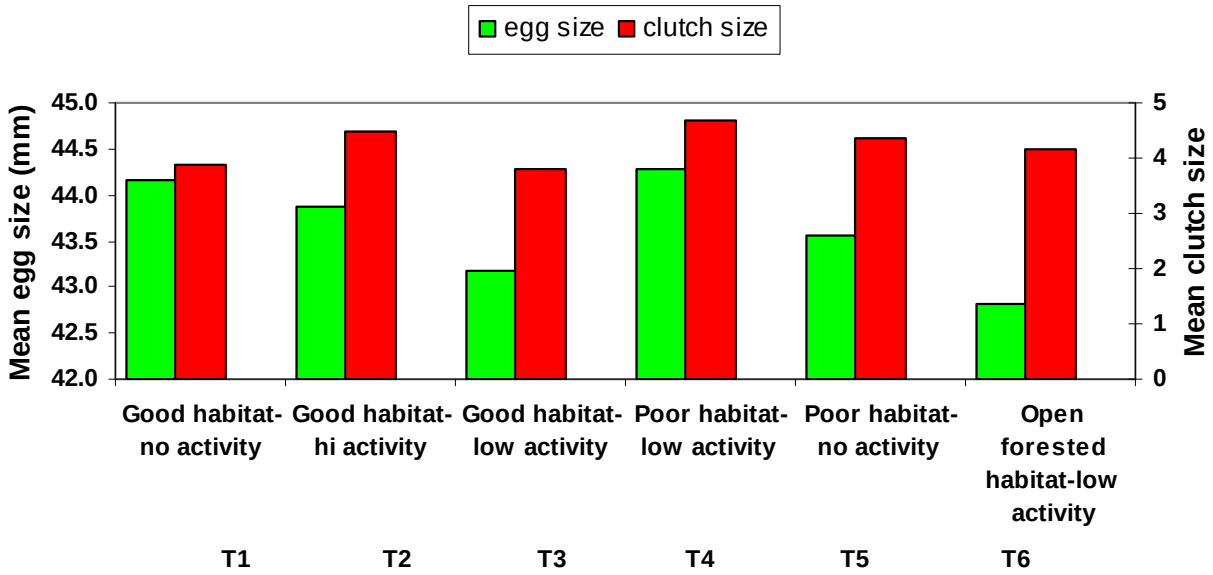

Figure 26. Indicators of reproductive competence in gopher tortoises as indicated by egg and clutch size at six experimental treatments (T1-T6) at Camp Shelby. Sample sizes are: T1 (N=8 clutches); T2 (N=25 clutches); T3 (N=5 clutches); T4 (N=3 clutches); T5 (N=11 clutches); and T6 (N=11 clutches).

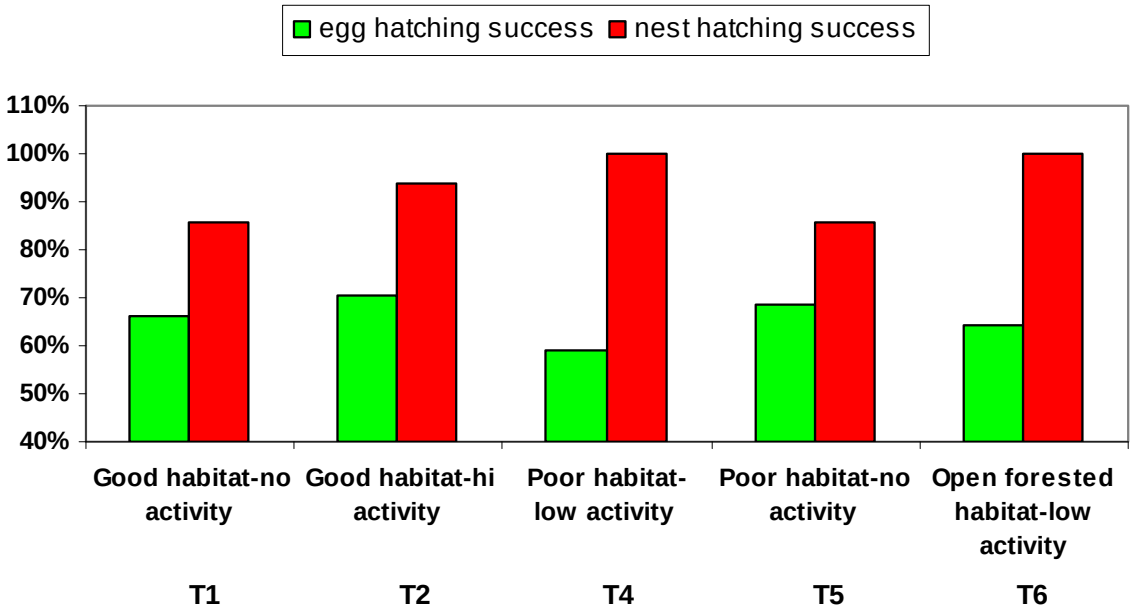

Figure 27. Indicators of reproductive competence as indicated by egg and nest hatching success in gopher tortoises at five experimental treatments (T1, T2, T4, T5, & T6) at Camp Shelby. Sample sizes are: T1 (N=7 clutches); T2 (N=15 clutches); T4 (N=2 clutches); T5 (N=7 clutches); and T6 (N=2 clutches).

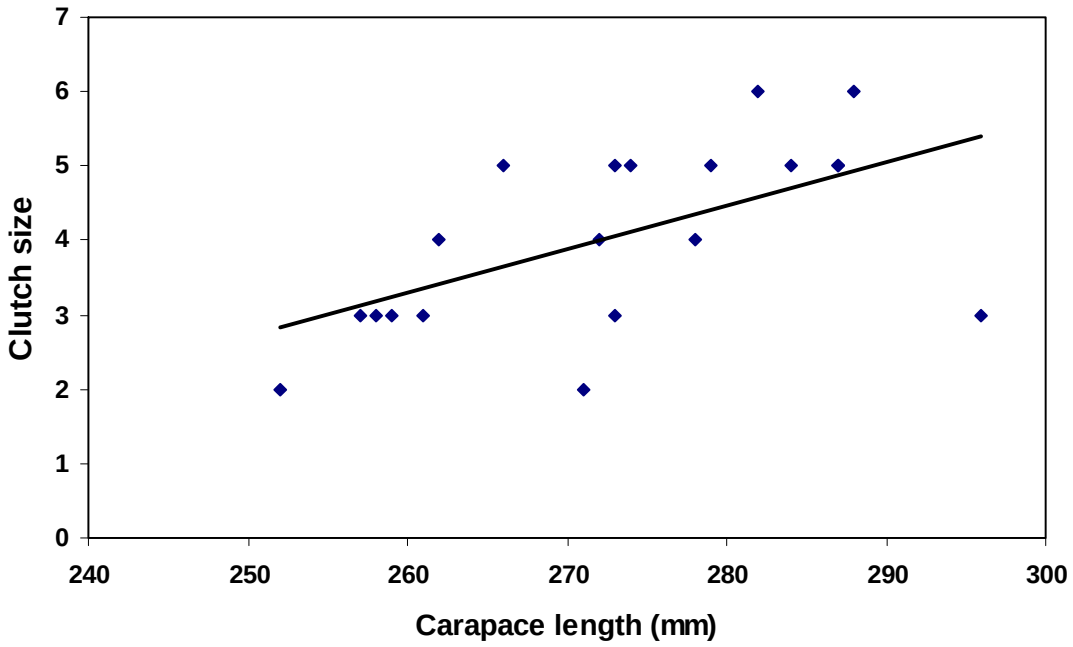

Figure 28. Relationship between clutch size and female body size (carapace length) for 20 female gopher tortoises at the Camp Shelby.

Population assessment

A population census of tortoises at each site was based on both the burrow scoping surveys (identification of active burrows) and the catch statistics from the live trap activities. Tortoise abundance as indicated by actual numbers captured in conjunction with assessment of active burrows at each site was highest at T6 with abundance at T2 being slightly higher than at T1 and T3-T5 (Fig. 29**)** and with T3 having the lowest population density. A relatively higher population abundance at T6 is not surprising since this treatment includes the tortoise refuge (T44E and T44W sites) where historically the abundance of tortoises has been reported to be relatively high.

In addition to an assessment of the population abundance and the number of active burrows at each site, an inventory of the number of inactive burrows was also taken at each site. The ratio of the number of active burrows to the number of inactive burrows at each site ranged from a high of 83% at FP 68 to a low of 39% at FP 507 (Table 3). There is no apparent pattern in the relationship of active to inactive burrows among sites; however, there does appear to be a pattern among some of the sites relative to the relationship between the number of active burrows and the actual number of tortoises at a site (Table 3). For example, sites that comprise Treatment 1 (the reference) had the highest ratio of active burrows to number of tortoises (0.92) while the sites comprising T6 had the lowest ratio of 0.67 (Table 3).

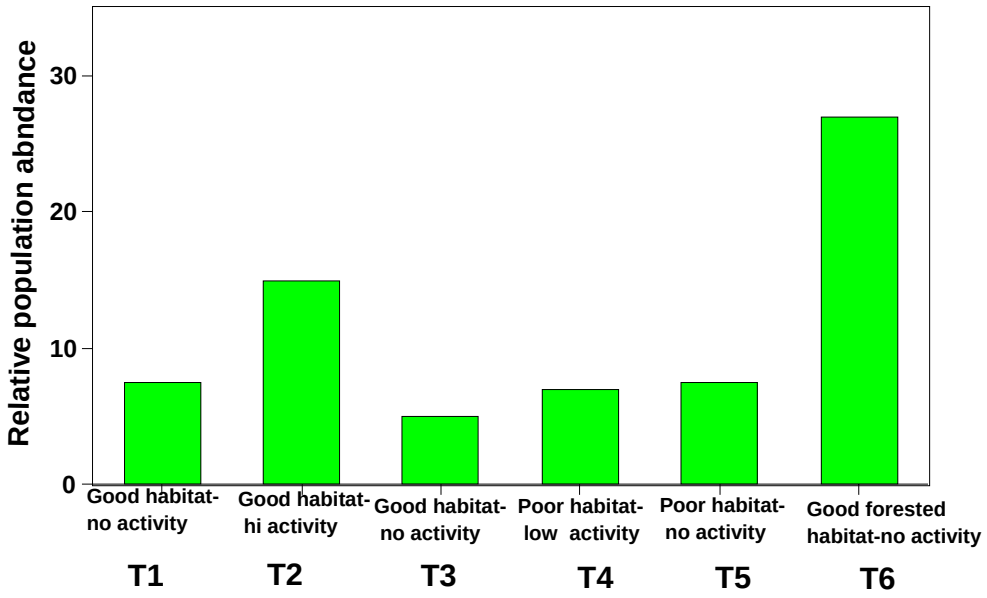

Figure 29. Population status as represented by relative abundance of gopher tortoises at the six experimental treatments (T1-T6).

Table 3. Population dynamics of the gopher tortoise at Camp Shelby relative to abundance and burrow status

Site	Treatment	# active burrows	# inactive burrows	% burrows active	# tortoises on site[1]	# tortoises / # of active burrows	Treatment mean
1	T1	12	9	57	10	0.83	
2	T1	5	5	50	5	1.00	**0.92**
121	T2	21	14	60	15	0.71	
140	T2	16	5	76	10	0.63	
68	T2	15	3	83	10	0.67	
OP-6	T2	23	23	50	25	1.09	**0.77**
507	T3	7	11	39	7	1.00	
136	T3	5	3	63	4	0.80	
72	T3	8	11	42	5	0.63	**0.81**
3	T4	11	9	55	7	0.64	
4	T4	8	7	53	6	0.75	
5	T4	11	16	41	7	0.64	**0.67**
6	T5	7	5	58	6	0.86	
7	T5	14	8	64	12	0.86	
8	T5	6	4	60	5	0.83	**0.85**
T44E	T6	41	45	48	30	0.73	
T44W	T6	59	33	64	33	0.56	
State lands	T6	26	20	57	19	0.73	**0.67**

[1]Represents the colony size present on the actual sample site including those active burrows present a few meters into the buffer zone surrounding each site

Integrated bioindicator analysis

Background - A canonical discriminant analysis procedure was used to assess the integrated health status of tortoises among the six experimental treatments at Camp Shelby. To examine the integrated responses of tortoises for each experimental treatment, the individual bioindicator variables were considered jointly within a multivariate context using this canonical discriminant analysis procedure (Adams et al. 1994). Canonical discriminant analysis generates new sets of variables that are linear combinations of the original bioindicators and account for most of the variation among treatment groups. This procedure provides a reduced set of canonical variables that are the most important and influential for differentiating responses among treatment groups. The greatest difference (or the highest discriminatory ability) among treatment groups is typically represented by the first canonical variate, the next greatest by the second canonical variate, etc. This procedure also identifies the canonical variate means that are most closely related among treatment groups. The 95% confidence regions (radii) were used to indicate the uncertainty associated with the estimated mean canonical variates from each treatment group. The diameter of each circle is a function of the variability structure of all bioindicator responses considered together and the associated sample size. The lower the sample size and the higher the variability, the greater the diameter of a circle. The center of each of the confidence regions is the mean value of the canonical variables. If circles overlap, then there is no statistically significant difference between treatment means. The position of each circle (treatment) in relation to the two canonical axes is only relative, but what is important is the relationship of the circles to each other. The linear statistical distances between the means of each circle (or each treatment) is measured quantitatively by the Mahalanobis distance and, the greater the linear distance between treatments (or midpoints of circles), the greater the dissimilarity of integrated health responses among treatment means.

Results -The greatest dissimilarity among treatments was between the reference (T1- purple circle) and the good forested-no activity treatment (T6- orange circle) (Fig. 30). The integrated health status of tortoises from the poor habitat treatments (T4 and T5) and from the good habitat treatments (T2 & T3) are more similar to the reference than are tortoises from treatment T6. Treatments T2 and T3 (represented by good habitat) are more similar to each other than they are to any of the other treatments suggesting that tortoises represented by these two treatments (good habitat and either high or low military activity) are similar in their health status compared to the tortoises from the other treatments. Tortoises from T4 and T5, represented by the poor habitat and little or no military activity, were also more similar to each other than the other treatments. In fact, because T5 and T4 intersect, and T3 and T2 intersect there is no significant difference between the treatments for each of these pairs (i.e., there is no significance between T4 and T5 and no difference between T2 and T3). Another interesting feature is that for each of these two pairs of treatments, the treatment with the higher activity (T2 in the case of T2 & T3, and T4 in the case of T4 &T5) are more dissimilar to the reference than are T3 and T5, respectively, illustrating that military activity has some small effect (but apparently not significant) on the integrated health response. As indicated by the linear statistical distances between treatment means (dashed lines in Fig. 30), both pairs of responses are approximately equidistant from the reference (T1).

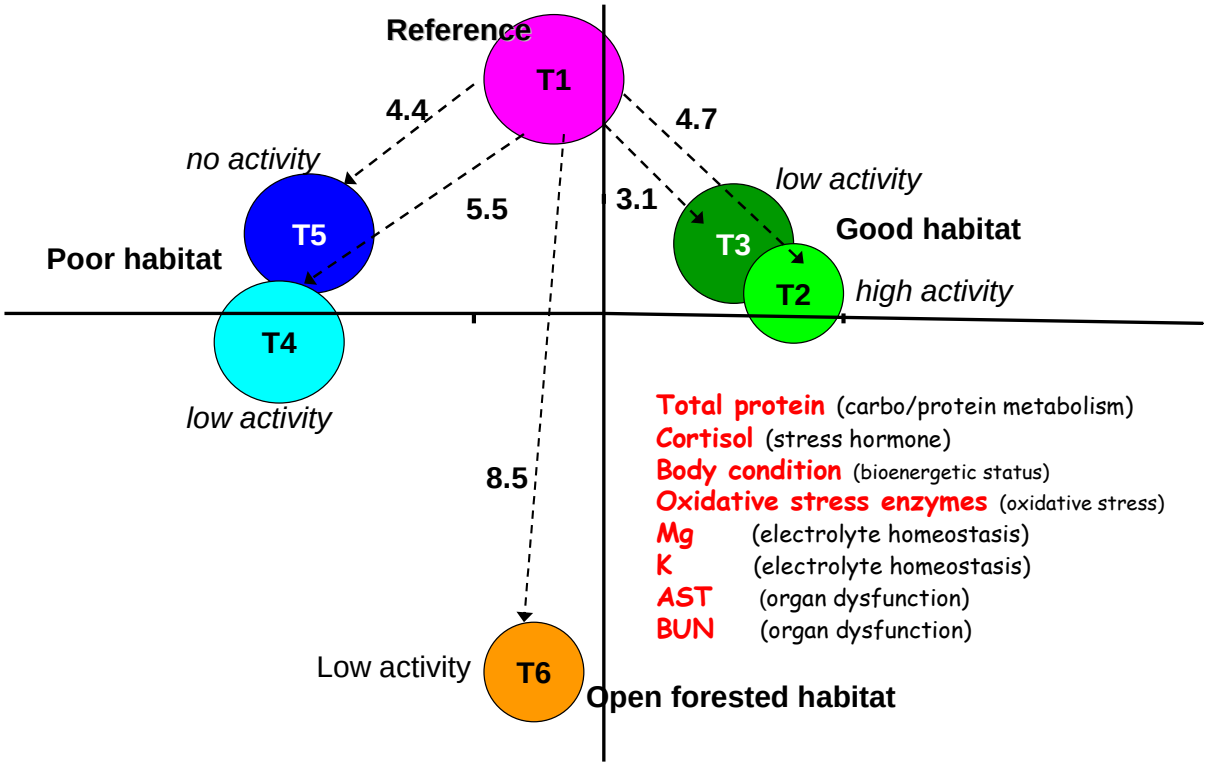

Figure 30. Integrated health responses of gopher tortoises at each of the six experimental treatments. Each circle represents the integrated health response and the 95% confidence radii of each response based on consideration on 20-25 individual bioindicators which were used within a canonical discriminant analysis procedure. Dashed lines represent a measure of the linear statistical distance between the mid-points of each circle. The greater the distance between mid-points of circles, the greater the difference in the integrated health response of tortoises among experimental treatments. Bioindicators listed in the lower right quadrant are the responses, in order of importance, that contributed the most to discrimination among all treatments. If circles overlap there is no significant difference between treatments.

A stepwise variable selection procedure was used to identify those variables that were the most important or influential for causing differentiation or separation among treatment groups. The stepwise variable selection procedure identified eight variables which were the most important or influential in causing separation or discrimination globally among the six experimental treatment groups (Fig. 30). These variables listed in decreasing order of importance are total serum protein, cortisol, body condition, an oxidation stress enzyme, serum magnesium and potassium, the transaminase enzyme AST (aspartate aminotransferase), and blood urea nitrogen. These eight variables represent six different physiological functional response groups including protein metabolism (total protein), a stress hormone (cortisol), an indicator of bioenergetic status (body condition), an indicator of oxidative stress (glutathione), two indicators of electrolyte homeostasis (magnesium and potassium), and two indicators of organ dysfunction (AST representing potential liver impairment and blood urea nitrogen representing potential kidney

impairment). Thus, of the approximately 25 individual variables that were included in the original canonical discriminant analysis, only about one-third of these were important in discriminating among treatment types and these eight variables basically represent five different physiological response groups that provide an integrated health profile of the tortoise.

Habitat analysis

Assessment of habitat quality and the type and level of habitat disturbance was determined using GIS telemetry in combination with direct plot-based measurement of key habitat and vegetation metrics. Amount of woody cover for each treatment within the 30m foraging zone (tortoises spend approximately 80% of time in this zone for daily activities) is shown in Fig. 31 as the percentage of trees (canopy) and tall shrubs (1-5m). Three treatments (T1-T3), represented by good habitat (grass or ruderal vegetation), had an average percent tree canopy cover of 25% and the two treatments characterized by poor habitat (T4 & T5) along with the good-forested habitat treatment (T6) had an average tree canopy index of 56%, over twice that of T1-T3. Similarity, for the good habitat treatments (T1-T3) the tall shrub category (1-5 m) averaged less than 10% and treatments T4-T5 averaged over 40%. Treatment T6, characterized by a mature long-leaf pine forest, had very little mid-story vegetation with the tall shrub category being only 5%.

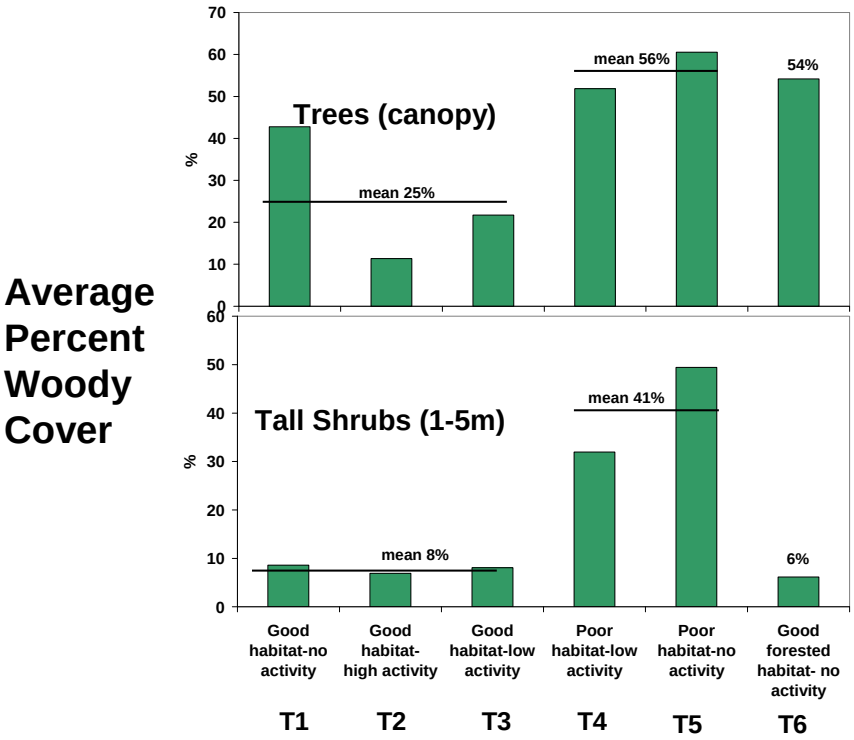

Figure 31. Vegetative survey results for the 30m tortoise forage area showing the percent of each experimental treatment that was composed of tree canopy cover and tall shrubs.

The preferred vegetation of tortoises, the herbaceous plants including graminoid species, is presented as percentage vegetative cover for each of the six treatments in Fig. 32 for the 30 m foraging zone. In contrast to the tree canopy and mid-story patterns seen in Fig. 31**,** the percentage of total herbaceous vegetation and graminoids averaged over 60 and 50%, respectively, for treatments T1-T3 (Fig. 32). These three treatments are characterized by less tree canopy and midstory vegetative cover and more by ruderal or grassland type vegetation, the preferred food of tortoises. Also in contrast to Fig. 31 **,** the percentage of herbaceous plants including graminoid species averaged between 10-20% at the two poor habitat treatments (T4 and T5) while T6, the mature longleaf pine forest with little mid-story, had a much higher percentage of herbaceous vegetation including graminoids.

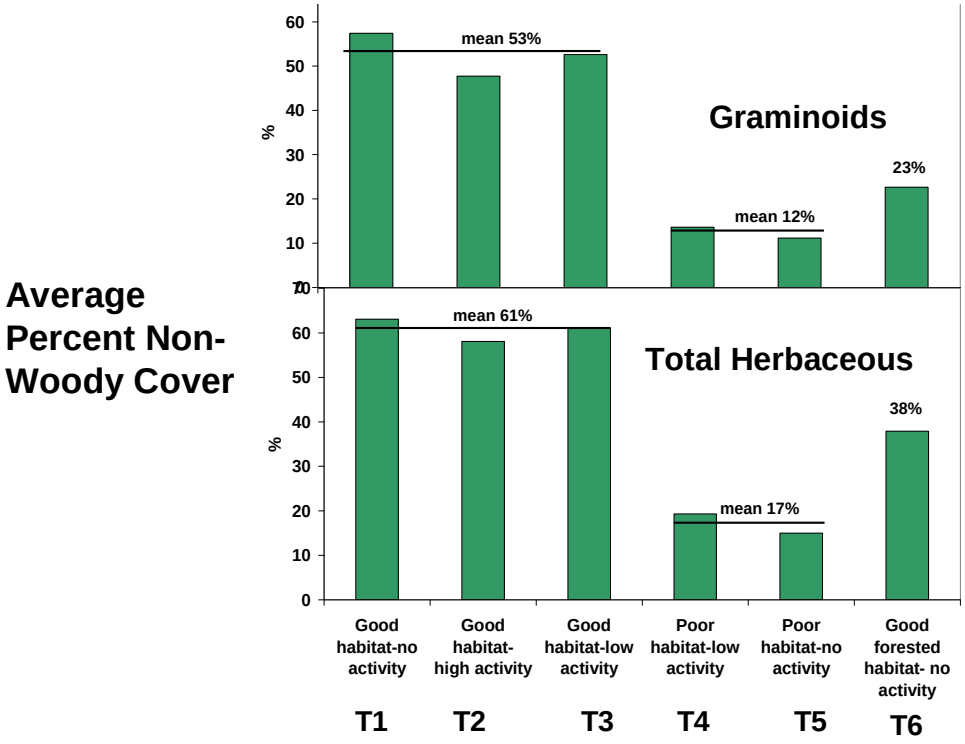

Figure 32. Vegetative survey results for the 30m tortoise forage area showing the percent of each experimental treatment that was composed of graminoid vegetation and total herbaceous plants, the preferred food of gopher tortoises.

The average amount of tall shrub cover (woody plants from 1-5 m in height) within the 200m buffer zone for each treatment is presented in Fig. 33**.** This zone, which extends out approximately 200 meters from the edges of the foraging area at each site (or from each burrow within each site), is basically outside the area of the primary tortoise foraging zone but does represent the area that tortoises may move between adjacent sites to mate, feed, or seek new burrow areas. Within this 200m buffer zone, woody cover is lowest at T6 (the treatment characterized by mature longleaf pines, relatively open canopy and little mid-story vegetation), highest at the two poor habitat treatments (T4 and T5), and intermediate at the three good habitat

treatments (T1-T3). Note some similarities between the percent shrub cover for the 200m buffer zone (Fig. 33) and for the tall shrubs at the 30 m preferred foraging zone (Fig. 31). In the case of the 30m foraging zone, shrubs were equally low at T1-T3 and at T6 and highest at T4 and T5. This pattern for tall shrubs is similar for the 200 meter buffer zone except T6 which has lower woody cover compared to T1-T3 than does the 30m foraging zone.

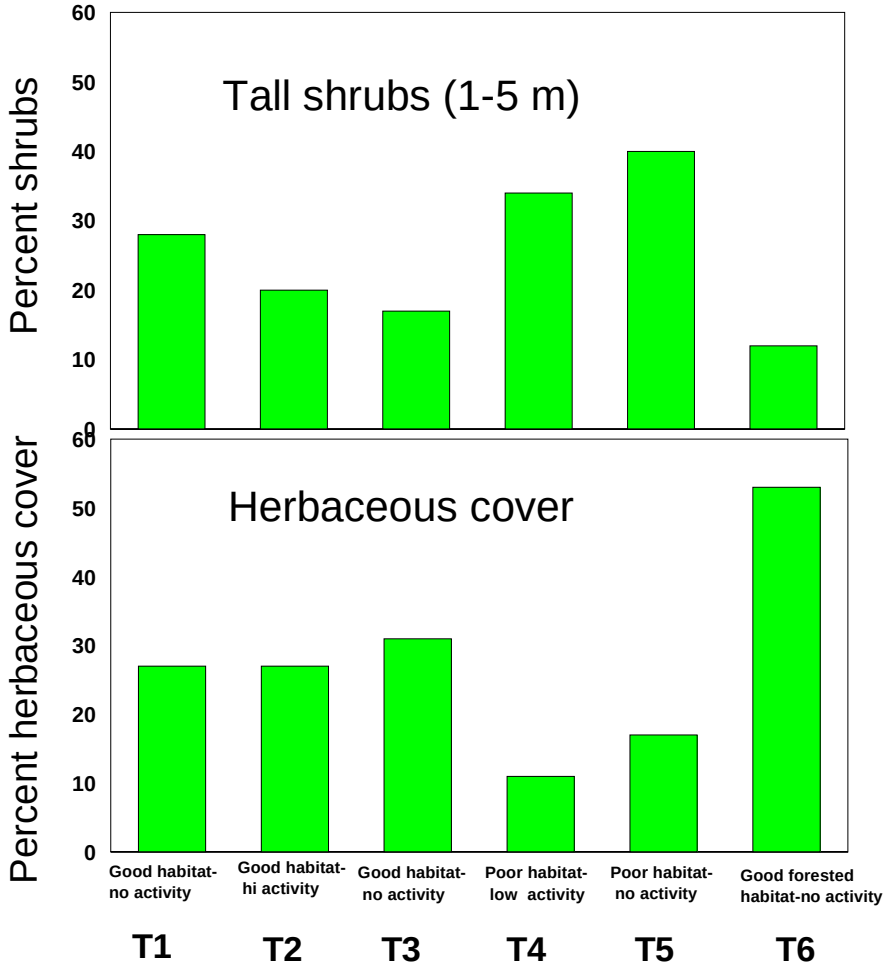

Figure 33. Vegetative survey results for the 200m buffer area showing the percent of each experimental treatment that was composed of tall shrubs and herbaceous cover.

Herbaceous cover within the 200m buffer zone for each treatment is also shown in Fig. 33. Within this 200m buffer zone herbaceous vegetation is much higher at T6 than at the other treatments with T4 and T5 being the lowest and T1-T3 being intermediate. There are some similarities between the percent herbaceous cover of the 200m buffer zone and for the 30 m preferred foraging zone (Figs. 33 and 32). In the case of the 200m buffer zone, herbaceous plants were lowest at T4 and T5 just as they were in the case of the 30m foraging area (Fig. 32). However, the largest difference in the herbaceous cover at T6 between the 30 and 200m zones

were that herbaceous plants were much more important in the 200m zone than they were within the 30m foraging zone.

Chemical (explosive residual) analysis

At Camp Shelby, soil samples for explosive residual analysis were collected from each treatment unit based on using 2-3 sampling grids of 2500m^2 for each treatment replicate. Within each 50 x 50 m sampling grid, approximately 100 surface core samples (2-3 cm surface depth) were collected. Thus, levels of explosive residuals in the soil for each treatment replicate were based on 200-300 increments or subsamples which were composited for analytical analysis. If surface water was present at any of the Camp Shelby sites, samples were also collected at these areas.

Even though analyses of explosive residuals were not a component of the original project plan for Camp Shelby, discussions early in the project with Tom Jenkins and Alan Hewitt convinced us that at least some preliminary chemical characterization surveys should be conducted to determine if gopher tortoises residing on active ranges could be exposed to energetic compounds. Results of soil and vegetation (grass included with soil corings) samples taken from several firing points at Camp Shelby and on the OP-6 demolition range indicate that NG, 2,4-DNT, and TNT are elevated above background levels at some sites (Fig. 34). Levels of explosive residuals in soil at the demolition range OP-6) are 10-20 times higher than explosive residuals at most firing points. Furthermore, energetic compounds in water from storm runoff sampled from the demolition site have levels of RDX and HMX up to 50 and 10 ug/l, respectively. Therefore, in addition to habitat disturbance at Camp Shelby, exposure to energetic compounds is another factor to consider in assessing the effects of military activities on gopher tortoises which reside on some of these ranges.

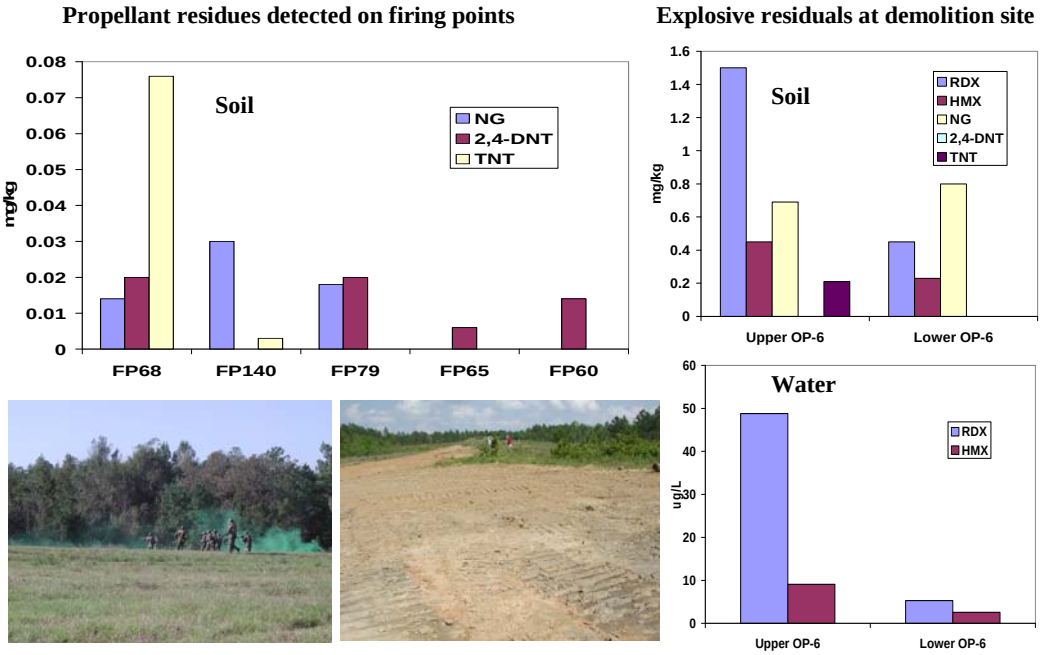

Figure 34. Levels of explosive residuals at several firing points (soil) and the OP-6 demolition range (soil and water) at Camp Shelby based on the chemical characterization studies.

Food habitats/diet quality

Diet analysis of gopher tortoises sampled at Camp Shelby revealed 51 species/genera of plant foliage material, 33 species/genera of plant seeds, and 26 species/genera of unknown plant seeds. Scat samples included roots, rhizomes, stems, prickles, thorns/spines, leaves, whole flowers, petals, fruits, and seeds, as well as insect parts, charred plant remains, wood, lichens, fungi, and mosses. The dominant components of almost all scats were graminoid (grasses) stems and leaves. Other common material included legume, pine, and oak foliage. Much of the legume foliage was apparently digested, but the graminoid, pine, and oak foliage was generally intact. Common fruits/seeds included *Rubus*, *Vaccinium*, *Dichanthelium/Panicum*, and *Plantago*.

The preferred food items of tortoises, the herbaceous plants including graminoids (grasses), legumes, and herbs (forbs), together constituted 90-95% of the diet at all treatments except at T5 and T6 where the herbaceous plants comprised about 80% of the total diet (Fig. 35). Grasses composed by far the largest percentage of the diet with tortoises from T1-T4 having from 70-80% of their total diet comprised of grasses, while grasses comprised about 50% of the diet for tortoises collected from T5 and T6. The relative importance of legumes and herbs (forbs) in the diet varied widely among treatments with T2 having the greatest percentage of legumes and T1, T5 and T6 having the highest percentage of herbs (forbs). Pine needles, which are a poor quality food item, comprised 10% or less of the diet for tortoises from T1-T5 but accounted for 20% of the diet for tortoises from T6. Tortoises from T2 and T3 had the lowest percentage of pine needles in the diet at less than 3%. It is interesting to note that tortoises from T6 which had the lowest quality diet because of the high percentage of pine needles also had the lowest percentage of the preferred grass in the diet.

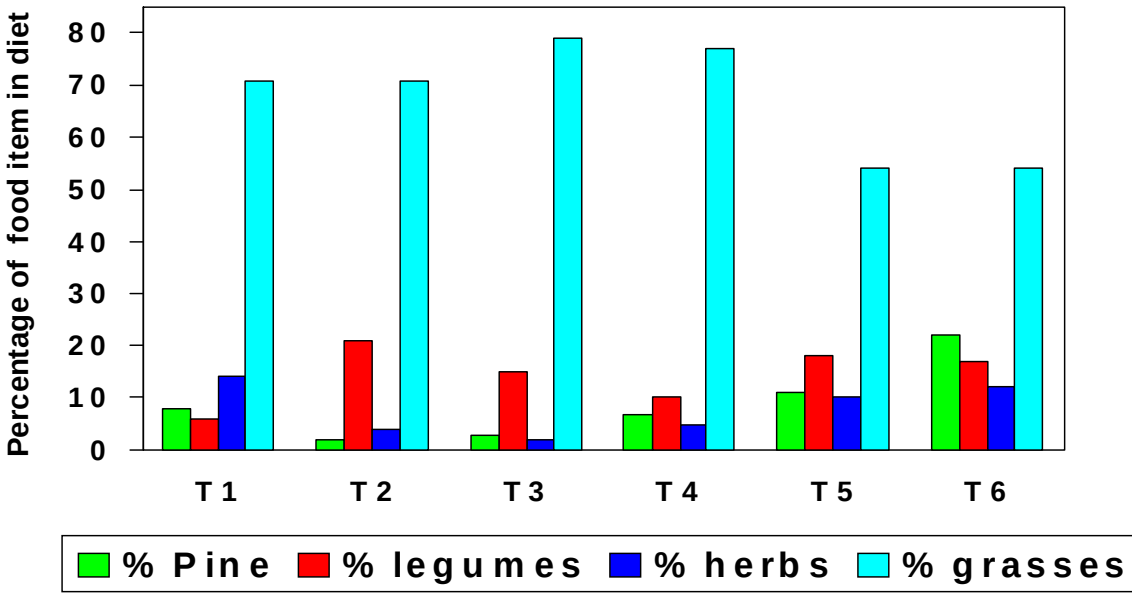

Figure 35. Percentage of major food items composing the diet of tortoises at the six experimental treatments at Camp Shelby.

The occurrence of the main food items in the diet along with the distribution of these items in the immediate environment of the tortoise reveal insights into the foraging habits of the tortoise. It has been previously established that the tortoise spends about 80% of its time foraging within a 30m radius of its burrow (see Technical Approach section, habitat characterization subsection). Figure 36 presents the percentage of the major food items in the tortoise diet compared to the percentage of these same food items present in the vicinity of tortoise burrows for each treatment. A comparison of the major food items in the diet and those same items available in the environment provides an indication of the foraging selectivity of these items by the tortoise for each treatment type.

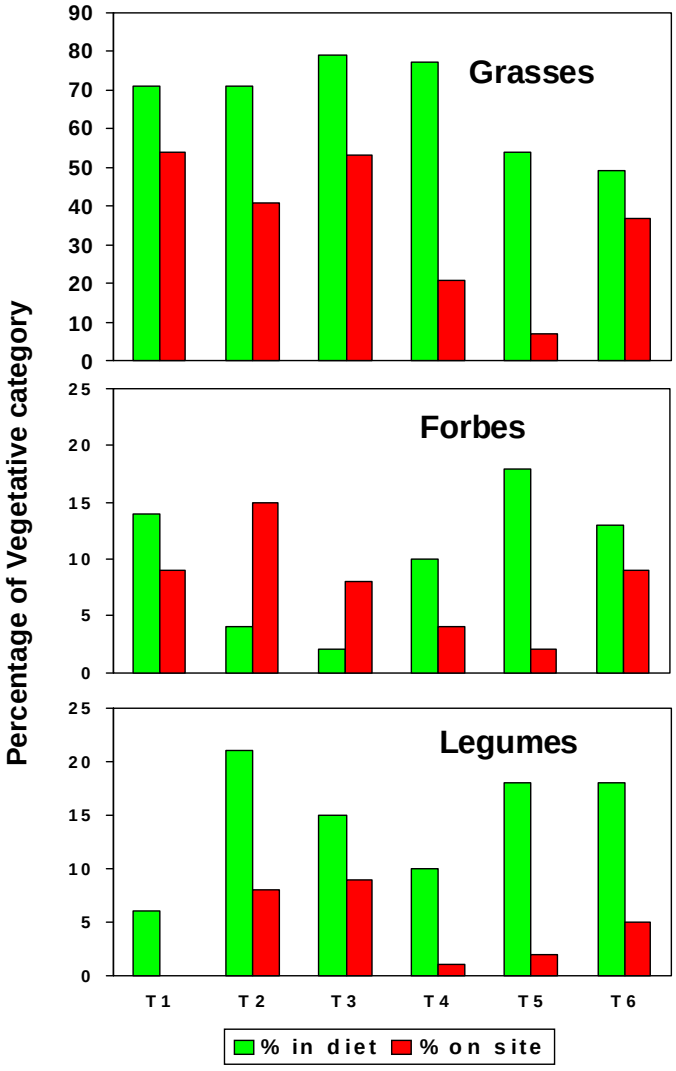

Figure 36. Percentages of major food items in the diet and within the 30m foraging area (determined from habitat characterization studies) for tortoises at the 6 experimental treatments at Camp Shelby.

Foraging selectivity by tortoises on their preferred food items is evident in Figure 36. Grasses, the dominant and preferred food item in the environment at most sites, is selected to a moderate degree by tortoises at T1-T3 and T6 while tortoises at T4 and T5, the two poor habitat sites, had a much high affinity for foraging on grasses. Also tortoises at T4 and T5 had a higher selective preference for forbs than they did at T1 and T6 with tortoises at T2 and T3 having a low or no affinity for forb selection. Legumes were highly preferred by tortoises at all treatments and particularly at T4 and T5, the poor habitat treatments.

Population and landscape genetics

This section consists of two components; the results of the population genetics analysis that quantifies the genetic diversity and variability of tortoises among colonies or metapopulations, and the landscape population genetics studies which are used to quantify those primary features of the landscape which could contribute to the observed genetic diversity for tortoises among the various groups.

Population genetics

Overall, genetic variation of gopher tortoises on Camp Shelby was low both base-wide (H_E = 0.220; allele richness = 27) and within the 25 sample sites (mean $H_E \pm 1$ SE = 0.209 $\pm$ 0.004, range = 0.163–0.246; mean allele richness $\pm$ 1 SE = 1.8 $\pm$ 0.04, range = 1.6 – 2.0) (Table 4). Genetic variation was greater in sites with good habitat (mean $H_E \pm 1$ SE = 0.211 $\pm$ 0.005; mean allele richness $\pm$ 1 SE = 1.9 $\pm$ 0.04) than in sites with poor habitat (mean $H_E \pm 1$ SE = 0.196 $\pm$ 0.006; mean allele richness $\pm$ 1 SE = 1.7 $\pm$ 0.02) (Table 1). However, there was no significant relationship between habitat quality and genetic variability when all samples (n = 6–40) were included in the analysis of heterozygosity ($t_{23} = 1.3$, $p = 0.22$) and allele richness ($t_{23} = 1.66$, $p = 0.11$) or when sample size was held constant across sites (n = 6) in the analyses of heterozygosity ($t_{23} = 1.77$, $p = 0.09$) and allele richness ($t_{23} = 0.65$, $p = 0.52$).

Genetic variability differed among the 6 treatment groups; however, differences most likely are due to idiosyncratic site differences rather than treatment differences. Generally, treatments composed of sites with greater population sizes had great genetic variability (Table 5). We found a significant difference across treatments for both population size ($F_{1,5} = 4.06$, $P = 0.028$) and sample size ($F_{1,5} = 3.35$, $P = 0.049$) where treatments 1, 2, and 6 had significantly greater population and sample size. When all samples were included, we found a significant difference among treatments for allele richness ($F_{1,5} = 4.50$, $P = 0.021$) but not H_E ($F_{1,5} = 2.41$, $P = 0.111$), where treatments 2 and 6 had greater allele richness. Differences for each measure of genetic variation were explained best by population size of sites within treatments (allele richness: $F_{1,14} = 35.76$, $p < 0.001$, $R^2 = 0.72$; H_E: $F_{1,14} = 8.19$, $p = 0.013$, $R^2 = 0.37$).

For analysis of all 25 sites (i.e., all sites sampled in the 2006 study plus sites from historic samples), genetic variation at each site was explained (weakly) by population size for heterozygosity ($F_{1,23} = 3.2$, $p = 0.09$, $R^2 = 0.12$) and significantly for allele richness ($F_{1,23} = 18.7$, $p < 0.001$, $R^2 = 0.45$) (Fig. 37 a-b). Sample size more accurately explained both heterozygosity ($F_{1,23} = 5.0$, $p = 0.03$, $R^2 = 0.18$) and allele richness ($F_{1,23} = 36.0$, $p < 0.0001$, $R^2 = 0.61$) (Fig. 37 c-d). Similar relationships were found within the 9 colony groups defined above. Genetic variation at each colony was explained (weakly) by population size for

heterozygosity ($F_{1,7}$ = 3.8, p = 0.09, R^2 = 0.35) and significantly for allele richness ($F_{1,7}$ = 8.2, p = 0.02, R^2 = 0.54) (Fig. 37 e-f). Sample size more accurately explained heterozygosity ($F_{1,7}$ = 15.2, p = 0.006, R^2 = 0.68) but was equally important to population size for explaining allele richness ($F_{1,7}$ = 7.2, p = 0.03, R^2 = 0.51) (Fig. 37 g-h).

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Table 4. Demographic and genetic data for 25 study sites where n > 5 individuals. Sites are grouped by habitat quality and site and mean data are presented for each expected heterozygosity and allele richness when all individual samples are included (full n columns) or when sample size is controlled (n = 6 columns).

	Population size	**Sample size**	**Habitat quality**	**full n H_E**	**full n # alleles**	**n = 6 H_E**	**n = 6 # alleles**
Cricket's Leap	8	8	Good	0.194	1.78	0.198	1.67
Deep Creek	15	11	Good	0.207	1.67	0.169	1.44
FP 121	20	20	Good	0.246	2.11	0.233	1.89
FP 140	15	15	Good	0.219	2.00	0.226	1.78
FP 507	7	6	Good	0.183	1.63	0.183	1.63
FP 65	7	8	Good	0.189	1.56	0.198	1.56
FP 68	16	17	Good	0.242	2.00	0.220	1.67
FP 72	14	15	Good	0.235	1.78	0.244	1.78
FP 79	9	6	Good	0.233	1.78	0.233	1.78
FP 91	6	6	Good	0.198	1.67	0.198	1.67
LRWMA	11	9	Good	0.163	2.00	0.192	1.89
Mars Hill	20	10	Good	0.191	1.78	0.207	1.67
MPRCH	40	40	Good	0.204	2.11	0.189	1.67
OP 6	25	12	Good	0.244	1.89	0.222	1.78
Range 18	20	7	Good	0.189	1.67	0.193	1.67
Range 45	35	6	Good	0.208	1.78	0.208	1.78
Site 1	25	21	Good	0.211	2.00	0.202	1.56
Site 2	7	7	Good	0.214	1.56	0.222	1.56
State Lands	25	19	Good	0.229	1.89	0.217	1.67
T 44 E	25	17	Good	0.199	2.11	0.222	1.78
T 44 W	30	20	Good	0.238	2.22	0.252	1.89
Site 3	10	7	Poor	0.182	1.67	0.182	1.67
Site 5	7	7	Poor	0.213	1.67	0.204	1.56
Site 6	6	6	Poor	0.192	1.75	0.192	1.75
Site 7	14	9	Poor	0.197	1.67	0.189	1.67
	Mean ± 1 SE Poor Habitat			**0.196 ± 0.006**	**1.69 ± 0.02**	**0.191 ± 0.005**	**1.66 ± 0.04**
	Mean ± 1 SE Good Habitat			**0.211 ± 0.005**	**1.85 ± 0.04**	**0.211 ± 0.005**	**1.70 ± 0.03**

Table 5. Summary genetic data for the six treatment groups. See Table 4 for data of individual sites within each treatment. Mean ± 1 SE data are presented for each of population size and sample size and for expected heterozygosity and allele richness when all individual samples are included (full n columns) and when sample size is controlled (n = 6 columns). For each population size, sample size, and # alleles (full n), the overall ANOVA was significant, and mean treatment values depicted in bold represent significantly greater values than all other treatments based on Fisher's PLSD multiple comparison method.

Treat-ment	Population size	Sample size	H_E (full n)	# alleles (full n)	H_E (n = 6)	# alleles (n = 6)
1	**16.0 ± 9.0**	**14.0 ± 7.0**	0.213 ± 0.002	1.78 ± 0.22	0.212 ± 0.010	1.56 ± 0.00
2	**19.0 ± 2.6**	**16.0 ± 1.9**	0.238 ± 0.007	**2.00 ± 0.05**	0.225 ± 0.003	1.78 ± 0.05
3	9.3 ± 2.3	9.7 ± 2.7	0.203 ± 0.016	1.65 ± 0.07	0.209 ± 0.018	1.65 ± 0.07
4	8.5 ± 1.5	7 ± 0.0	0.198 ± 0.016	1.67 ± 0.00	0.193 ±0.011	1.61 ± 0.06
5	10.0 ± 4.0	7.5 ± 1.5	0.194 ± 0.002	1.71 ± 0.04	0.225 ± 0.001	1.71 ± 0.04
6	**26.7 ± 1.7**	**18.7 ± 0.9**	0.222 ± 0.012	**2.07 ± 0.10**	0.230 ± 0.011	1.78 ± 0.06

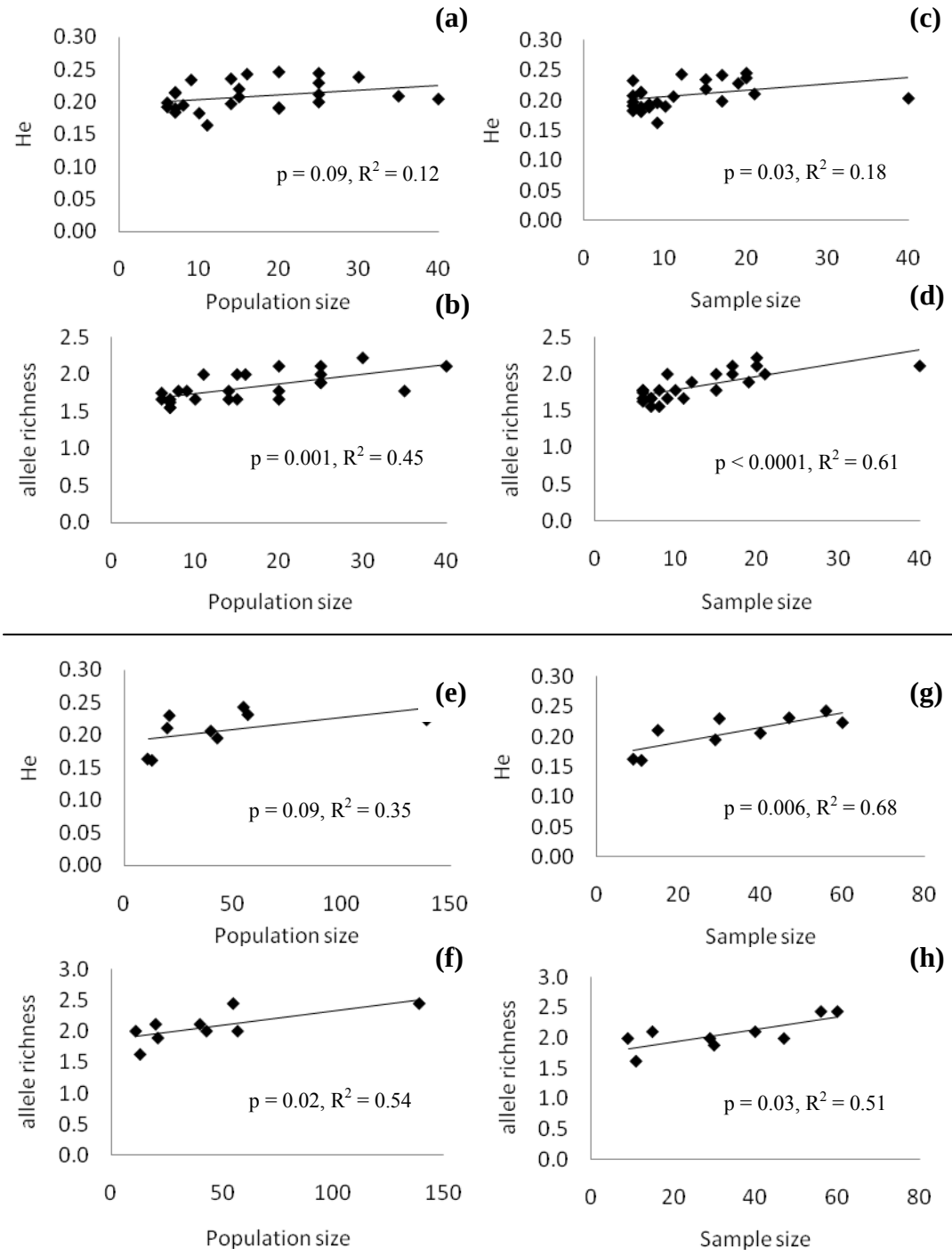


Figure 37. Bivariate plots depicting the relationship between genetic variation (He = expected heterozygosity or allele richness) and sample size or population size for all 25 sites (a-d) and for 9 colony groups (e-h). Statistics from regression analyses are provided for each plot. See text for statistical methodology.

Landscape population genetics

Structuring of genetic variation across the spatial scale of Camp Shelby was statistically significant but had a rather weak association (F_{ST} = 0.025; 95% confidence interval = 0.017–0.030). Populations (sites) were separated by 1.5–45 km between pairs, and pairwise F_{ST} values ranged from 0–0.25. Genetic differences between populations (i.e., pairwise F_{ST}) were not explained by the geographic distances between them as would be predicted by an isolation-by-distance (IBD) model (P = 0.38). However, there was a slight positive trend for IBD (Fig. 38A) indicating a weak relationship between genetic and geographical distance between sites.

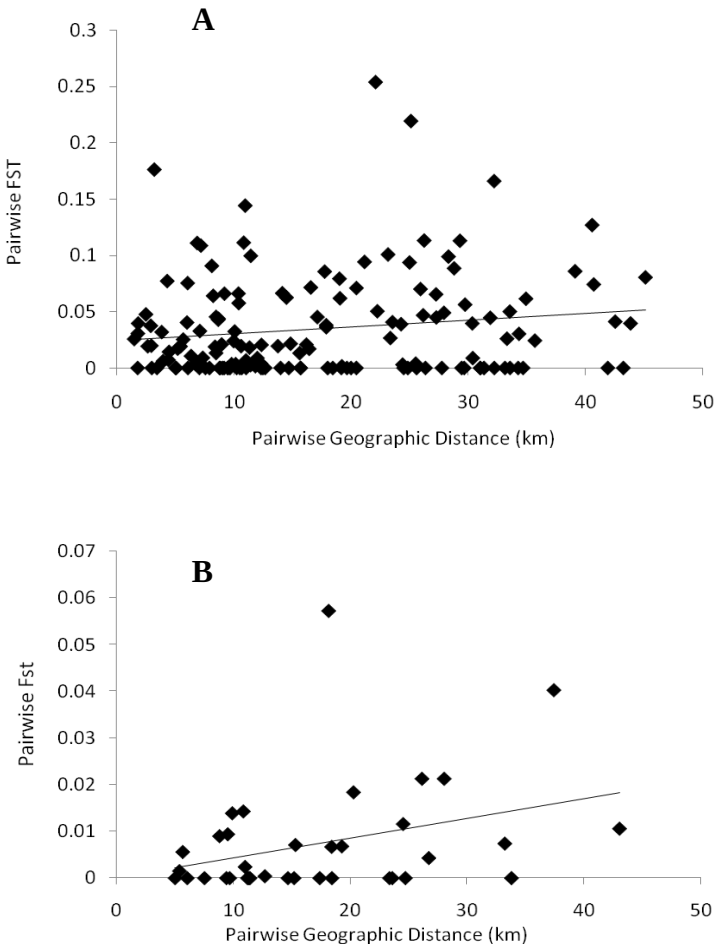


Figure 38. Bivariate plot depicting the relationship between geographic distance and pairwise genetic distance (F_{ST}) between all pairs of sample sites (A) and between all pairs of grouped (colony) populations (B).

Based on the 9 colony groups, significant structuring of genetic variation across the colonies was not detected (F_{ST} = 0.004; 95% confidence interval = 0.0–0.010). Colony groups had 5.0–43 km between pairs, and pairwise F_{ST} values ranged from 0–0.06. Although site-wide non-significant population structure was found, there was a relatively high positive relationship between pairwise geographic and genetic distances between groups (Fig. 38B). However, this relationship was only marginally significant (Mantel test; P = 0.07). Genetic differences between colony groups were not explained by the geographic distances between them as would be predicted by IBD.

Because genetic differentiation between colony groups was weak and non-significant, the analysis of genetic differentiation was restricted to descriptive statistics for pairs of colony groups as a function of the major habitat and landscape features within the 2 km-wide corridor. Quantification of the major habitat features between each pair of colony groups is presented in Table 6. A Pearson correlation coefficient was calculated and bivariate plots were examined for each landscape feature (Table 6) predicted to impact genetic distribution of tortoises across the base compared to pairwise F_{ST} values between colony groups. Trends for weak positive relationships were found between genetic differentiation (F_{ST}) and for % favorable habitat (Pearson r = 0.192) and for number of burrows (Pearson r = 0.228). No obvious trend were observed for number of stream segments (Pearson r = 0.018), for % swamp bottom (Pearson r = 0.051), and for geographic distance (Pearson r = -0.073). There was a weak negative relationship between genetic diversity and km of roads (-0.153) (Fig. 39).

Table 6. Quantification of various habitat features in 2 km-wide corridors between pairs of site-group clusters (colonies)

Site-group comparison	Corridor[1] area (km^2)	Favorable[2] habitat (km^2)	Unfavorable[3] habitat (km^2)	% favorable habitat	% unfavorable habitat	Streams (km)	Km streams/ km^2 corridor	Roads (km)	No burrows in corridor	# burrows/ km^2 corridor
1_2	22.7	12.8	7.4	63.5	36.5	37.4	1.6	85.6	125	5.5
1_3	30.6	20.9	9.7	68.3	31.7	60.5	2.0	172.9	355	11.6
1_4	36.8	20.3	5.6	55.3	15.2	72.4	2.0	64.2	1204	47.9
1_5	39.9	26.0	10.6	70.9	29.1	73.9	1.9	101.9	817	22.3
1_6	53.5	32.2	21.2	60.3	39.7	98.2	1.8	140.5	695	13.0
1_7	58.4	38.7	15.9	70.8	29.2	103.4	1.8	115.4	1096	23.0
2_3	15.5	13.5	2.0	87.1	12.9	33.6	2.2	38.4	424	27.3
2_4	19.8	16.8	2.9	85.2	14.8	54.5	2.8	31.3	932	66.4
2_5	20.6	16.9	3.7	82.1	17.8	46.3	2.2	39.6	721	15.2
2_6	33.9	21.1	12.8	62.2	37.8	72.0	2.1	57.6	424	12.5
2_7	38.6	29.2	9.4	75.6	24.3	76.2	2.0	63.2	932	24.2
3_4	26.3	21.9	4.4	83.3	16.5	65.7	2.5	28.3	559	31.4
3_5	19.9	15.4	4.6	77.1	22.9	46.8	2.3	19.3	633	31.9
3_6	26.9	18.1	8.9	67.0	33.1	49.6	1.8	31.1	197	7.3
3_7	35.0	24.3	10.6	69.6	30.4	57.1	1.6	44.2	461	13.2
4_5	14.7	11.7	3.0	79.5	20.5	36.0	2.4	16.0	803	62.5
4_6	31.5	22.3	9.2	70.9	29.1	63.4	2.0	36.6	1097	35.6
5_6	20.3	13.9	6.4	68.4	31.6	34.7	1.7	24.5	457	22.5
5_7	21.5	15.4	6.1	71.5	28.5	36.7	1.7	26.5	489	22.7
6_7	16.6	12.4	4.2	74.9	25.1	36.1	2.2	16.4	72	4.3
6_8	51.4	41.4	9.8	80.5	19.1	106.8	2.1	39.0	62	1.2
7_8	40.1	26.2	6.3	80.5	19.5	66.9	1.7	38.5	153	3.8
9_7	27.9	19.9	7.0	73.8	26.2	64.9	2.3	20.1	82	2.9
9_8	17.6	13.9	2.4	85.1	14.9	31.9	1.8	16.3	144	8.2

[1] Corridor is 2 km wide connecting midpoints of each site-group pair.

[2] Favorable area habitat metrics include longleaf pine forests, pine plantation/old field, grassland, roads, and number of burrows.

[3] Unfavorable area habitat metrics include physical structures, pine flatwoods, swamp and bottomland, and water bodies.

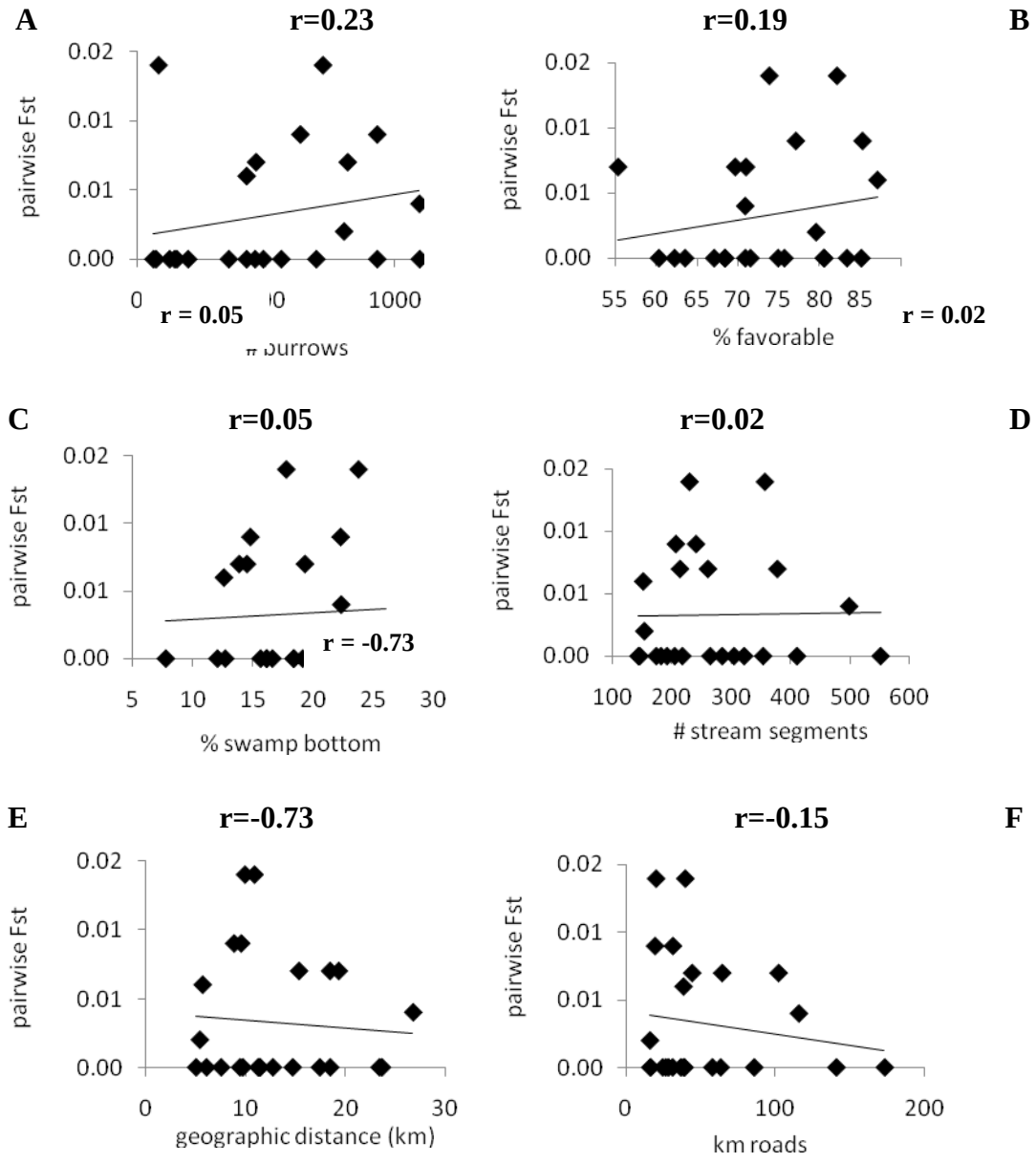


Figure 39. Bivariate plots of the relationship between genetic differentiation (pairwise F_{ST}) and between two colony groups and each of (a) number of tortoise burrows, (b) % favorable habitat, (c) % swamp bottom, (d) number of stream segments, (e) geographic distance, and (f) km of roads. Pearson correlation coefficient provided within each plot. Pairwise F_{ST} is represented on all y-axes.

Gopher Frogs-Eglin AFB

Chemical characterization

Water samples taken from one of the high disturbance areas (the "cat's eye" on the C52N range) indicated that the levels of some explosive residuals were elevated above background and are also similar to values of explosive residuals found in ground water of test wells on the C52N range which are monitored semi-annually by DoD. Levels of RDX and HMX in the ground water from test wells on this site were 2.1 and 0.3 ug/l, respectively (Eglin AFB 2003). The test wells are located immediately adjacent to the main activity area of the "cat's eye" and the surface water samples were collected 300-400m from this area. Since levels of energetic compounds in the ground water samples are similar to levels in surface water samples, the primary source of these compounds to the surface water ponds and seeps is probably the "cat's eye" area. To confirm this, maps of groundwater hydrology show that the general flow of the shallow subsurface water from the "cat's eye" is toward the wetland areas where our surface water samples were collected (Eglin AFB 2003). Analysis of water samples taken from a pond at the B70 range also indicate detectible levels of some energetic compounds, and water samples taken from a water retention basin on the C74 range immediately adjacent to the rocket testing facility also had detectible but relatively low levels of RDX. Soil and water samples taken in the "C62N area during 2003 by another investigator indicate detectible levels of RDX, HMX, NG, and TNT (Jenkins and Foote, unpublished data).

Habitat characterization

A wide range of pond habitat types and level of military activity were characterized at Eglin AFB during multiple field trips in the first year of this project. Ten separate treatment areas (4 high, 3 intermediate, and 3 low military activity) representing groupings of 2-8 ponded areas, were surveyed (Table 7). Not all proposed sites were surveyed in late 2005, as some sites were proposed for further characterization during the future frog collection dates in 2006 and 2007. Gross pond characteristics and morphology were defined using a combination of GIS tools and direct measurement and field observations. High amounts of precipitation in the late spring of 2005 helped define likely flooding and definition of shore vegetation zones for the fall habitat surveys. On average, each treatment area represented approximately 7 acres of pond habitat (range 3.4 acres to 15.5 acres) with 1277 m (range 1109-1509) of pond perimeter for potential frog breeding (Table 4).

Pond depth and surface area were taken on the habitat survey dates, but were unlikely to be representative of more flooded pond conditions when the frogs breed. Some ponds were dry on the survey dates or had little water. Soon after these initial habitat characterization studies were conducted, the panhandle area of Florida including Eglin AFB experienced an extended drought period, while not as severe as 2006-2008, is still occurring. When water was present in ponds during the initial survey period in 2005, water quality metrics such as clarity/turbidity, temperature, DO, conductivity, and pH were taken. In general, ponds were clear with turbidity at or near zero, temperatures were in the 27-28^{o} C range (typically of Sept-Oct in this area), DO was 7-8 mg/L, pH slightly acidic to highly acidic (4-6.9), and conductivity generally from 0.01-0.02 ms/cm^{3}.

Table 7. Designated pond sampling sites and treatment areas at Eglin AFB based on evaluation of military activity, pond size, shore perimeter distances, and terrestrial zones of pond groupings

Range	Area	Pond ID	Military activity	Pond size (ac)	Pond perimeter (m)	Total pond perimeter per area (m)	Terrestrial range (acres/area)
C-52	North	C52N-1	High	1.0	330	1245	207
C-52	North	C52N-2	High	0.1	90	1245	207
C-52	North	C52N-3	High	0.2	180	1245	207
C-52	North	C52N-4	High	1.0	277	1245	207
C-52	North	C52N-5	High	1.0	277	1245	207
C-52	North	C52N-6	High	0.1	90	1245	207
C-52	Cats Eye	C52CE-7	High	0.1	77	1220	153
C-52	Cats Eye	C52CE-8	High	0.1	70	1220	153
C-52	Cats Eye	C52CE-9	High	0.1	67	1220	153
C-52	Cats Eye	C52CE-10	High	0.2	98	1220	153
C-52	Cats Eye	C52CE-11	High	1.0	277	1220	153
C-52	Cats Eye	C52CE-12	High	1.0	277	1220	153
C-52	Cats Eye	C52CE-13	High	1.0	277	1220	153
C-52	Cats Eye	C52CE-14	High	0.1	77	1220	153
C-74	Sled Track	C74-1	High	1.5	383	1509	279
C-74	Sled Track	C74-2	High	0.8	285	1509	279
C-74	Sled Track	C74-3	High	0.5	200	1509	279
C-74	Sled Track	C74-4	High	0.6	207	1509	279
C-74	Sled Track	C74-38	High	2.8	434	1509	279
B-70	West	B70-1	High	1.7	367	1293	149
B-70	West	B70-2	High	2.8	495	1293	149
B-70	West	B70-3	High	2.6	431	1293	149
B-70	West	B70-4	Intermediate	1.5	346	1239	94
B-70	West	B70-100	Intermediate	13.9	893	1239	147
C-52	C	C52C-15	Intermediate	0.7	211	1143	296
C-52	C	C52C-16	Intermediate	3.8	538	1143	296
C-52	C	C52C-17	Intermediate	2.5	394	1143	296
C-52	A	C52A-19	Intermediate	2.4	403	1495	183
C-52	A	C52A-20	Intermediate	1.1	244	1495	183
C-52	A	C52A-21	Intermediate	0.9	217	1495	183
C-52	A	C52A-87	Intermediate	2.8	439	1495	183
C-52	A	C52A-87b	Intermediate	0.6	192	1495	183
OFF	EG99 Area	REF-99	Low	2.6	527	1220	110
OFF	EG99 Area	REF-01	Low	0.7	202	1220	110
OFF	EG99 Area	REF-162	Low	4.1	491	1220	110
OFF	Roberts Pond	REF-07	Low	5.3	697	1296	252
OFF	Roberts Pond	REF-09	Low	1.3	321	1296	252
OFF	Roberts Pond	REF-176	Low	1.2	278	1296	252
OFF	Wildcat Area	REF-37	Low	2.8	643	1109	134
OFF	Wildcat Area	REF-10	Low	3.3	466	1109	134

Eglin vegetation surveys were largely semi-quantitative to qualitative in nature. Random transect and plot-based surveys categorized the major plant species and percent cover within the pond zone. Reference ponds (off range, low military activity areas in the EG99 pond area, Wildcat area, and near Roberts Pond) were representative of natural depressional ponds ideally suited for gopher frogs and many other amphibian species. These round or oval, low-slope, shallow ponds typically offered sturdy, vertical graminoid vegetation as support for frog egg masses (Palis 1998; Bailey 1990). Pond centers were often open or with submerged graminoid species, with an extensive pond edge community of emergent wetland grasses and rushes (*Juncus* spp). Dominant grass taxa included broomsedge bluestem (*Andropogon virginicus*), switchgrass (*Panicum virgatum*), rosette grass (*Dichanthelium* spp.), and rice cutgrass (*Leersi*a spp.). Ponds deemed to be of "intermediate" military activity exhibited many of the same vegetative characteristics, with a greater propensity of land disturbance, including vehicle disturbance, evidence of earth moving such as berms and partial pond filling, presence of exploded ordnance, and/or cratering. Ponds in the intermediate category were all located on-range (C52 A, C52C, and the west section of B70), but distant from the most active current testing zones. With greater land disturbance, plants were absent in some areas, or there was more extensive coverage of less desirable plant species (for example, the dominance of soft-leaved submerged and floating leaf species instead of graminoid). Edge and terrestrial habitat may also be less open and less graminoid-dominated, with greater percentage of species in scrub habitat including saw palmetto (*Serranoa repens*), smooth sumac (*Rhus glabra*), shiny blueberry (*Vaccinium myrsinites*), sand live oak (*Quercus geminata*), and slash pine (*Pinus elliottii*).

In general, high military activity areas, located within C52 North, the "Cat's Eye" (C52N range), C-74 sled-track, and B70 test sites, were areas with the poorest habitat for gopher frogs. Many of these aquatic sites did not have typical round depressional features, were relatively small, and steeper banked. Shallow zones of emergent graminoid growth, best suited for supporting frog egg masses, were relatively uncommon or event absent at some sites. Atypical sites included swamp areas (with extensive pine and more acidic aquatic conditions), baygall seeps and bogs, and water-filled bomb craters (especially within C52N and the Cats Eye). Man made depressions were also surveyed, including runoff areas next to the C74 sled-track and concrete-lined testing basins on B70. The plant communities at these sites often consisted of disturbance-adapted, nonnative species, and were highly variable in percent coverage and species composition depending on the site. With few exceptions, most of the aquatic areas within the most disturbed parts of the testing ranges were unlikely to be suitable habitat for gopher frogs. However, leopard frogs were deemed to be an appropriate surrogate for gopher frogs for this study, and this species was likely to be adaptable to almost any pond habitat without fish (and in fact were observed at some high-military impacted sites during the habitat surveys).

DISCUSSION

Individual Health Responses

Overall condition - An important factor to consider for explaining the lower BCI at T6 compared to the other treatments is that this tortoise refuge area represented by the T44E and T44W sites has a higher abundance of tortoises compared to other sites and treatments (see Fig. 29), possibly resulting in increased interspecific competition for available food and other resources. Also, there could be increased behavioral interactions which could result in higher levels of stress and therefore lower bioenergetic efficiency (and ultimately lower body condition) in individuals at these sites. Higher energetic demands resulting from higher activity metabolism from activities such as increased foraging, movement, and defense of territories would necessarily result in less stored lipid reserves and therefore lower body condition.

Hematological response - Tortoises from T2 and T6 had lower specific immune responses (lymphocytes) but higher phygocytic ability (heterophils) compared to the reference (T1). The reasons for this situation are not readily apparent because one of the treatments (T2) has high military activity while the other (T6) has no activity but both treatments are characterized by good quality habitat. Perhaps tortoises from T2 and T6 have a higher incidence of infection (as indicated by the higher levels of heterophils) but their immune systems haven't been challenged by environmental stressors to such a level that triggers a specific immune response. One intriguing similarity is that sites comprising both treatments T2 and T6 have been heavily studied by natural resource biologists in recent years, meaning resident tortoises there have been trapped and handled more than those at other sites. It is also interesting to note that for tortoises at T1 the elevated levels of lymphocytes and the reduced levels of heterophils appear to be related to the lower baseline levels of cortisol (Figs. 25 & 23). Cortisol is a known immunosupressant, particularly for the specific immune system (i.e., lymphocytes) (Hou et al. 1999; Saha et al. 2004), and the higher lymphocyte levels at T1 (Fig. 25) would be consistent with our finding of lower baseline cortisol levels (Fig. 23) because these lower levels of cortisol would not suppress lymphocyte production of tortoises at T1.

Association of bioindicators across treatments - Individual bioindicator responses that were relatively unique for tortoises from T2 and T3 (the good habitat and military activity treatments) and that displayed some significant deviation from the reference condition (T1) were total serum protein and glucose for T3 (carbohydrate/protein metabolism), AST and LDH (organ dysfunction indicators) for T2 and T3, gluthathione for T2 (oxidative stress enzyme), and the stress hormone cortisol for T2 and T3. Individual bioindicator responses that were relatively unique for tortoises from T4 and T5 (poor quality habitat and little or no military activity) and displayed some significant deviation from the reference were lipid peroxidation (T4) and the serum electrolytes of Ca, Mg, and P at T4 and T5. Decreased levels of serum protein and glucose, two organ dysfunction indicators, and the stress hormone cortisol appear to be those bioindicators that are relatively unique to these two treatment types (T4 and T5) and, therefore, these particular types of responses may reflect effects of habitat quality on tortoise health. Additionally, those individual bioindicators that appear to be more reflective of effects of habitat on tortoise health are the oxidative stress enzymes and serum electrolytes (Figs. 20 & 22). For those individual response parameters that are more reflective of military activity, the indicators of

carbohydrate/protein metabolism, organ dysfunction, and stress hormones (cortisol) reflect stress responses which are typically associated with anthropogenic stressors such as chemical contamination, noise, and ground disturbance (Figs. 19, 21, & 23). For those individual response parameters that are more indicative of habitat quality, indicators related to lipid peroxidation (oxidative stress) and levels of essential nutrients in the body are more typically associated with the quality of the diet.

Reproductive Competence

The reproductive status of tortoises at Camp Shelby appears to differ from that reported previously for tortoises at Camp Shelby and also for tortoises throughout other parts of its range in the Southeastern US (Table 8). The average clutch size of tortoises across Camp Shelby in 2006-2008 was 4.5 eggs per gravid female, comparable to the 4.8 average clutch size determined through burrow observations by Epperson and Heise (2003) in a previous investigation of tortoise reproduction at Camp Shelby. The 4.5 average clutch size is much lower than the range of average clutch sizes (8.6 to 12.6 eggs per gravid female) reported over several years for resident gopher tortoises in Florida (Small and Macdonald 2001) and on the low end of the range of clutch sizes (3.8-7.0) surveyed for tortoises over other areas in the Southeastern U.S. (Diemer and Moore 1994; Landers et al. 1980, 1982; McLaughlin 1990; Wright 1982) (Table 8). Tortoises from the far eastern and western parts of their range tend to be smaller resulting, therefore, in smaller clutch sizes. It should also be noted that the average and range of clutch sizes for relocated tortoises in the Florida study were smaller than the resident tortoises (4.8 to 9.7 eggs per gravid female), although still larger than observed at Camp Shelby.

Table 8. Reproductive status of the gopher tortoise across its range in the Southeastern U.S.

Location	Average clutch size	Original source
Florida[1]	5.8	Diemer and Moore (1994)
Florida[1]	7.0	McLaughlin (1990)
Florida[1]	8.6 - 12.6	Small and MacDonald (2001)
Georgia[1]	7.0	Landers et al. (1980, 1982)
South Carolina[1]	3.8	Wright (1982)
Mississippi (Camp Shelby)	4.8	Epperson and Heise (2003)
Mississippi (Camp Shelby)	4.5	This study (lab incubation only)

[1] From Small and McDonald (2001).

Greater reproductive effort is generally expressed as larger clutch size (as opposed to egg size) in chelonians, since egg size tends to be fairly uniform across populations (Iverson and Smith 1993). Although not statistically significant, the differences in clutch size for tortoises at Camp Shelby were explained more by site than by treatment, habitat, or military activity. Since gopher tortoises are thought to exhibit indeterminate growth (J. C. Callaway, unpubl. data), and clutch size in this study was found to have a significant positive linear relationship with female body size (Fig. 28), this fails to support the theory that female gopher tortoises senesce.

The percentage of gravid females (50%) across Camp Shelby falls well within a broad range (22 % to 78%) reported for gopher tortoises studied over multiple years at a site in Florida (Small and MacDonald 2001). Even though the percentage of gravid females at Camp Shelby falls within the range reported for other geographical areas in the SE-US, the percentage of gravid females at some of the sites could be artificially low particularly for tortoises at those sites that were sampled later in the reproductive season (such as at T6) where individuals may have deposited their eggs before they were trapped. In this situation, eggs would not have appeared on the radiographs and a greater percentage of females at this site would have therefore been recorded as non-gravid, when, in fact, their eggs could have been deposed before capture. For example, several sites were not trapped until June 2006, and of the 50 nests located on Camp Shelby between 2006-2008, 50% (25) were oviposited in the month of May. Therefore, the 50% value for how many radiographed tortoises were gravid should be used as a minimum value. The special reproductive studies conducted in spring/summer 2008 were designed to circumvent this issue of capturing late ovipositing females.

Although egg size (and egg weight) may not be as important as clutch size in determining reproductive competence, there is definitive data to support the importance of egg quality in the life history of the tortoise. Since egg size and weight have a strong positive relationship with corresponding hatchling size and weight (M. Hinderliter, in prep.), these reproductive-related metrics can be important predictors of a population's reproductive competence. Studies of desert tortoises (*G. agassizii*) have shown that increased juvenile survivorship is based more on the size of the animals than their age (K. A. Nagy, pers. comm.); therefore larger hatchlings would have a better chance of reaching reproductive maturity than their smaller cohorts. Interestingly, although Camp Shelby gopher tortoises have smaller average clutch sizes than those in Florida (Table 7), both egg size and egg weight are generally larger. From this study, average egg size was found to be 43.7 mm, while in Florida average egg size has been documented as 40.6 mm (Small and Macdonald 2001) and 40.2 mm (Demuth 2001). Similarly, average egg weight in this study was found to be 45.6 g, while in Florida average egg weight has been documented as 37.7-41.0 g (Demuth 2001; Iverson 1980; Linley 1987; Small and Macdonald 2001). Perhaps gopher tortoises in the western part of the range over time have sacrificed larger clutch sizes to focus their reproductive effort in favor of larger and higher quality eggs. Although not statistically significant, the differences in egg size from this study were explained more by site than by treatment, habitat, or military activity. The differences in egg size by site approached significance ($P = 0.06$), with the lowest sizes found at T-44 (treatment T6), and the highest found at Firing Points 140 and 68 (treatment T2). Differences in egg weight were explained more by habitat than any other factor, and approached significance ($P = 0.09$). Lowest egg weights were found at forested sites, while highest weights were documented at the grassland sites such as firing points 68 and 140.

Another indicator of reproductive competence is hatching success. Hatching success documented at Camp Shelby during this study (70%) is lower than has been recorded previously in Florida and Georgia (Table 9), although it falls well within the range of values documented for the species, and is much higher than has been previously reported on Camp Shelby (29%) by Epperson and Heise (2003). However, hatching success from this study is from artificially incubated eggs, while those recorded by Epperson and Heise were from wild nests. Hatching success values were almost identical when compared by site, by treatment, by habitat, and by military activity.

Table 9. Comparison of hatching success reported for gopher tortoises across its range. Hatching success determined for this study is the average across all treatments (combinations of habitat and training activity).

Location	Hatching success	Original source
Florida	73%	Butler and Hull (1996)
Florida	57%	Pike and Seigel (2006)
Florida	67% and 97%	Smith (1995)
Georgia	86%	Landers et al. (1980)
Louisiana	65%	Hurley (1993)
Mississippi	50%	Brode (1959)
Mississippi (Camp Shelby)	29%	Epperson and Heise (2003)
Mississippi (Camp Shelby)	70%	This study (lab incubation only)

Even though there were no statistically significant differences among treatments for any of the reproductive metrics measured (Figs. 26 and 27), clutch size, and to some extent hatching success, indicate that the reproductive competence of tortoises at Camp Shelby could be somewhat impaired relative to tortoises across other parts of its range. Also, even though several of the biochemical and physiological indicators demonstrated differences among treatments, the various reproductive metrics such as egg hatching success, nest hatching success, clutch size, and egg size did not show similar differences among treatments. This finding is not surprising since biological response parameters such as reproductive-related metrics are characterized by long-term response modes which integrate many features of the environment (such as food availability, climatic variables, intraspecific competition) over long time scales. In contrast, the rapidly- responding biochemical and physiological parameters are sensitive and shorter-term bioindicators which reflect the effects of environmental factors over much shorter time scales. Thus, because of the relative "insensitivity" of longer-term integrative responses such as reproductive metrics, these responses, in themselves, are not the preferred "early warning" or sensitive response indicators to environmental factors such as military activity or habitat quality. Therefore, the sensitive (i.e., biochemical, physiological) and short-term response indicators should be used along with the longer-term but more integrative (i.e., reproductive) indicators to effectively assess the effects (or potential effects) of environmental factors such as level of military activity and habitat on the health and fitness of wildlife species of concern at military installations.

Population Fitness

For tortoises from some of the treatment groups there appears to be a pattern between population density and other indictors of tortoise health. Even though the population abundance was highest at T6 compared to the other treatments (Fig. 29), the egg size was lowest (Fig. 26), the body condition was lowest (Fig. 23), cortisol was highest (Fig. 23), and the ratio of active burrows to actual numbers of tortoises present on a site was also the lowest (Table 3). The higher population density at T6 may have been responsible for an increased in intraspecific competition for food, preferred habitat, and for mates resulting in response of the various indicators of reduced population fitness. McRae et al. (1981) found that intraspecific interactions of tortoises increased with density causing individuals to increase movement (and therefore active metabolism) and construct new burrows. The high percentage of poor quality food in the diet (Fig. 36) is highly confounding since these sites have a low percentage of shrubs and high percentage of herbaceous groundcover (Fig. 29), so even with increased competition there should be ample high-quality forage.

Tortoises from T1 had the highest ratio of active burrows to actual number of tortoises while individuals from T4 and T6 had the lowest ratio (Table 3). A high number of tortoises compared to the number of active burrows could indicate that individuals were moving around less and were less engaged in re-locating and constructing new burrows. This higher ratio, which is evident for T1 tortoises, further suggest that individuals from this treatment spent less energy in activity metabolism, and therefore more energy was available for fat storage, maintenance of body condition, etc. The lower ratio of number of tortoises to number of active burrows may indicate that tortoises from the T44 refuge area (T6) and T4 areas were moving around more, re-locating and constructing burrows more often, and spending more energy in activity metabolism, etc. than those tortoises from T1. Mushinsky and McCoy (1994) have suggested that large numbers of active and inactive burrows, relative to the actual number of tortoises present at a site, may signal a stressed population or at least characterize a population where intraspecific competition is increased due to increased densities (McRae et al. 1981). While such patterns are not as evident for tortoises from Treatments 2, 3 and 5, such relationships are clearly indicated for tortoises from T1 vs. T6 where there are obvious differences in several indicators of tortoise health which could be directly related to population density and thus increased intraspecific competition.

Integrated Health Responses

A canonical discriminant analysis procedure was used to assess the integrated health status of tortoises among the six experimental treatments and to identify which particular bioindicator variables were most responsible for discriminating among treatments. Because the treatments pairs T2 & T3 and T4 & T5 are basically equidistant from the reference treatment (T1) (Fig 30), this pattern suggests that both habitat quality and military activity may both be important in influencing the health and condition of tortoises at Camp Shelby. Additionally, since treatment pair T4 & T5 are statistically similar to each other and treatment pair T2 & T3 are statistically similar to each other (because they overlap), there are no differences in the particular bioindicators which separate treatments within pairs (i.e., separate T2 from T3 or separate T4

from T5) (Fig. 30). Because of these similarities, the bioindicators that are responsible for separation of T4 & T5 from T2 & T3 are, therefore, the same variables that separate (1) T5 from T3, (2) T5 from T2, (3) T4 from T2, and (4) T4 from T3. Variables that are primarily responsible for separating T6 from T4 & T5, T6 from T2 & T3, and T6 from T1 are different combinations of the 8 variables listed in the lower right quadrant of Fig. 30. For example, body condition and cortisol are the two most important bioindicators responsible for separation of T6 from T1, while serum electrolytes (Mg and K) and the oxidative stress enzymes are the two most important variables separating T4 & T5 from T6. Oxidative stress enzymes, body condition, and egg size are the variables most responsible for separation of T6 from T2 & T3.

Since the experimental treatments of this project were based on the incorporation of both habitat-related and military-related components into the design, some of the eight main variables that were most important in discriminating among treatments in Fig. 30 were differentially more reflective of habitat effects and other variables were more representative of military-related effects. For example, the organ dysfunction, protein metabolism, and stress hormone variables appear to be more closely associated with military activity and the electrolyte homeostasis, oxidative stress, and bioenergetic-related variables appear to be more reflective of habitat status of the tortoise (Fig. 30). Because of the nature of the experimental design, only the treatment pairs T2 & T3 and T4 & T5 had variables which could be definitively identified as either related to habitat or to training. This situation occurs because the treatment pair T2 & T3 included sites that were all good habitats but had some level of military activity while the treatment pair T4 & T5 included sites that were characterized by poor habitat and either low or no military activity (Fig. 30). Thus, separation of these two treatment pairs was based almost exclusively on habitat type and level of military activity. The other possible treatment combinations (i.e., T6 vs. T1, T6 vs. T2 & T3, etc) do not include both habitat and level of activity as the two main treatment factors, therefore the variables that discriminate between these other treatment combinations would not be as distinct and clear cut as the T2 & T3 vs. the T4 & T5 comparison and would necessarily include a mixture of both habitat-related variables and activity-related variables.

Weight-of-Evidence Approach for Assessing Tortoise Health

The complexity of ecological systems, their inherent high variability, and the influence of multiple and interacting environmental factors suggests that no single measure (or perhaps even a few measures) is adequate for assessing the effects of multiple stressors on the health or fitness of wildlife species (and especially sensitive or TER-S) and for establishing the mechanistic basis of these effects. An appropriate suite of endpoints including multiple lines of evidence (weight-of -evidence) is required for determining the effects of these stressors and for better understanding the underlying cause or mechanistic basis of observed effects (Adams et al. 2002, Adams 2005, Attrill and Depledge 1997, Hodson 2002, Galloway et al. 2004). In many instances, simply documenting that a change has occurred in a system or measuring such a change with a few response parameters may not be adequate to assess causal relationships between environmental stressors and health effects on organisms. Over-reliance on any one, or even a few, indicators for assessing effects and their underlying cause(s) can result in environmental management and regulation that is not only less accurate but could be either under or over-protective of natural resources (Yoder and Rankin 1998).

In developing a weight-of-evidence approach for assessing the effects of multiple environmental factors on the health or fitness of gopher tortoises, both the individual bioindicator responses and the integrated health responses were used together in a joint assessment. For the individual bioindicator responses, each of the stress response categories (listed in the first subsection of this Discussion section) were ranked from 1-6, with a ranking of 1.0 representing the best health and a ranking of 6.0 representing the poorest health for that particular stress response among the six treatments (Table 10). For example, the body condition index of tortoises at T6 received a ranking of "6" because tortoises from this treatment had the lowest body condition index compared to the other five treatments, and tortoises from T1 received a ranking of "1" because these tortoises had the highest condition (Table 10) among treatments. Likewise, each treatment received a ranking of 1-6 for each of the other stress response categories. Ranks for all stress response categories were summed over each treatment and an overall rank assigned to each treatment based on the value of the summed ranks. Thus, the final ranking or "health scores" represent the relative health status of tortoises at each treatment. Considering all the individual bioindicator responses together, these results clearly show that tortoises from T1 had the best overall health and tortoises from T6 had the lowest overall health. The health status of tortoises from T3 and T5 were similar (ranking = 30) and had the next highest health status while the health of tortoises from T2 and T4 (rankings = 34 and 35, respectively) were also similar but had an even lower health ranking (Table 10).

Table 10. Ranking of health status of gopher tortoise among treatments based on inclusion of all the functional bioindicator responses and on the integrated discriminant analysis. Rankings range from 1-6 with 1 representing the best health and 6 being the poorest health.

Functional response	**Rankings**					
	Treat 1	**Treat 2**	**Treat 3**	**Treat 4**	**Treat 5**	**Treat 6**
DNA damage	1	2	4	4	4	6
Electrolyte homeostasis[1]	1	3	3	5	5	4
Carbohydrate – protein metabolism[2]	4	3	1	5.5	5.5	2
Organ dysfunction[3]	1.5	6	5	3	1.5	4
Stress hormone	1	5	3.5	2	3.5	6
Hematology[4]	1	5.5	3.5	3.5	2	5.5
Oxidative stress[5]	5	1	4	3	2	6
Bacterial killing (immune)	4	6	2	4	4	1
Body condition	1	2.5	4	5	2.5	6
Sum of ranks	**19.5**	**34**	**30**	**35**	**30**	**40.5**
Ranking based on all functional responses listed above	**1**	**4.5**	**2.5**	**4.5**	**2.5**	**6**
Ranking based on discriminant analysis[6]	**1**[7]	**4**	**2**	**5**	**3**	**6**

[1] Average for ranks of Ca, Mg, Na, K, P.
[2] Average for ranks of total protein, albumin, glucose.
[3] Average for ranks of BUN, uric acid, AST, CK, LDH, AP.
[4] Average for ranks of lymphocytes and heterophils.
[5] Average for ranks of lipid peroxidase and glutathione.
[6] Rankings based on the linear distance of each treatment mean (mid-point of each circle) from T1
[7] Assumes that T1 has the highest health based on Fig. 30.

The second important component for applying a weight-of-evidence approach in assessing effects of multiple environmental factors on tortoise health is to include the results of the integrated health responses presented earlier in this section. Figure 30 shows the integrated health status of tortoises among the six experimental treatments. The dashed lines connecting pairs of circles represent a measure of the linear statistical distance (i.e., the Mahalanobis distance) between the mid-points of each circle. The greater the distances between the midpoints of these circles, the greater the difference in the integrated health response among treatment pairs. Using these quantitative measures of linear distances between the reference (T1) and each of the other 5 treatments (Fig. 30), then tortoises from T3 are in the best health compared to the reference followed in decreasing order of health by tortoises from T5, T2, T4, and T6, respectively (Table 10).

In comparing (1) the ranking of final scores among treatment generated from the summation of ranks for the individual functional response categories, and (2) those ranking provided by the integrated health response analysis, it is evident that both approaches (the individual function response rankings and the integrated health response ranking) provide very similar results relative to the health status of tortoises among treatments. In both approaches, tortoises from T6 have the poorest health followed in decreasing order of health by T2 and T4 which are similar to each other and T3 and T5 which are also similar to each other.

Based on the results of this weight-of-evidence approach to assess the health of tortoises among the six treatments, it appears that both habitat quality and military activity both influence the health of tortoises at Camp Shelby. This is the same conclusion reached using the canonical discriminant analysis procedure to assess the integrated health responses of tortoises among treatments. This conclusion resulting from the weight-of-evidence analysis, is based on the fact that health scores of tortoises over the 6 treatments varied as a function of both habitat quality and level of military activity and there was no consistent pattern of increasing or decreasing health scores as a function of habitat quality or level of military activity. For example, tortoises from treatment T2 (good habitat, high military activity) had the second best health score, T3 characterized by good habitat and low activity had the next highest health score, and T4 with poor habitat and low activity was ranked as the forth highest health score. In other words, there was no consistent pattern in either habitat quality or level of military activity in influencing health status among treatments; however, both factors influence, to some degree, the health of tortoises at Camp Shelby.

The three treatments (T2, T3, and T4) that include some level of military activity are characterized by intermediate levels of health or condition (Table 10). These results suggest that military activity at Camp Shelby is having some effect on tortoise health. Studies by Guyer et al. (1996) found that tracked vehicle activity at Fort Benning, Georgia could impact tortoises directly by restricting the amount of their surface activity, or indirectly, by making it more difficult for tortoises to find food or mates. The amount of time spent by a tortoise outside its burrow reflects time used for foraging, basking, and social behaviors. Tracked vehicles have an indirect effect on tortoises by possibly forcing them to forage longer for limited vegetation or to search longer for mates due to altered sex ratios. The short-term responses in behavior associated with activity by tracked vehicles by Guyer et al. (1996) study suggest that size of home range was increased and that longer and perhaps earlier times of activity were necessary to traverse

their home ranges ultimately resulting in decreased tortoise fitness. Tracked vehicle activity appears to impact females more than males and this pattern is consistent with failure of reproduction on impacted sites. These results are similar to those reported by Diemer (1992) on a site with poor vegetation relative to a site with more vegetation illustrating that poor habitat can not only affect tortoise health, but effects can be similar to those resulting from military activity. Another indicator that reproductive competence and population fitness may be compromised in areas with increased training activities and or poor habitat is that the ratio of inactive to active burrows may be higher than in areas where stress levels are lower (Mushinsky and McCoy 1994).

Habitat Assessment

The primary purpose of the habitat assessment and the vegetative analysis is to evaluate possible relationships between tortoise health and the type and quality of the habitat at each sample site. Within the 30m foraging zone, the three treatment groups which are characterized primarily by a high percentage of tree canopy cover and tall shrubs (T4 & T5) had very little preferred habitat for the tortoise (Fig. 31) which might help explain, in part, why tortoises at these treatment sites were in generally poorer health than those tortoises at sites characterized by a much higher percentage of herbaceous vegetation. As an example of a direct link between habitat quality and tortoise health, the significantly lower levels of essential nutrients such as calcium, magnesium, and phosphorus in tortoises from the two poor habitat treatments (T4 and T5) indicates a poor quality diet that is basically lacking in these nutrients (Fig. 22). The good forested habitat treatment (T6), even though characterized by a tree canopy cover similar in some ways to T4 and T5, has a percentage of tall shrubs (mid-story) that is very low compared to T4 and T5, allowing sufficient sunlight to the forest floor to support an adequate base of herbaceous vegetation, the preferred food of tortoises. Tortoises from the good habitat treatments (T1-T3, T6) had significantly elevated levels of these essential nutrients (Fig. 22) possibly reflecting a higher quality diet. This relationship between habitat quality and nutritional status can also be seen from Fig. 32 where percentages of grasses, forbs, and legumes at T1-T3 were more prevalent in the environment than at T4 and T5. The influence of poor habitat quality may also be reflected in the response of other bioindicators such as the low levels of both glucose and total protein at T4 and T5, and the relatively low levels of cortisol (e.g., poor nutrition influences the ability to mount an adequate immune response in some situations).

While the type of vegetative habitat within the 30m foraging zone is important in the life history of the tortoise from the perspective of nutritional and physiological (health), data from the 200m zone reflects less about nutritional and food quality issues but more about habitat status and how it serves as either a barrier or a relatively unobstructed corridor for movement of tortoises between preferred feeding or breeding areas. Movement corridors are important for tortoises in seeking new burrows, mates, or feeding areas between adjacent sites. In addition, such corridors helps to maintain genetic diversity by minimizing isolation which can lead to genetic sinks and lower population genetic diversity (Schwartz and Karl 2006). If buffer areas are characterized by thick vegetation, most of which is not utilized for foraging, then such areas could potentially discourage movement of tortoises between areas and tortoises could become somewhat geographically and genetically isolated resulting in lower population genetic fitness and individual health.

As evidence by the percentage of woody cover and herbaceous vegetation for the 200m buffer zones for each treatment, T4 and T5 potentially have the greatest "barrier" for expansion of tortoise colonies, extended movement, and provision of preferred forage. In contrast, the 200m buffer zone for T6, or the tortoise refuge area, is characterized by mature longleaf pine forests and appears to have a rather unlimited area for unrestricted movement (as indicated in Fig. 33 by the low percentage of shrubs). Also the T6 area has a high relative abundance of preferred herbaceous vegetation for foraging by tortoises in proximity to their burrows (Fig. 32). Thus, treatments T4 and T5 have the highest relative potential impedance to longer-range movement of tortoises, followed by the buffer zones around the firing points (T1-T3) which presents an intermediate level of impedance to any long-range movement. In contrast, the buffer zone associated with T6 has the lowest impedance to movement. Apparently the areas represented by the buffer zones for T1-T3 have been maintained periodically by controlled fire regimes helping to keep, therefore, the canopy somewhat open while T4 and T5, the poor habitat areas, have had very little or no fire maintenance within the past several years. Much of the tortoise refuge area (T6) is regularly maintained with controlled fires by the US Forest Service resulting in a minimum midstory and abundance of preferred herbaceous vegetation.

Habitat type and landscape features influence the movement and home range of tortoises. Movement and home range of tortoises increased as the amount of herbaceous biomass decreased in an area (Auffenberg and Iverson 1979). Gopher tortoises pack burrows more closely and relocate burrows less frequently in habitats with relatively dense herbaceous ground cover than in areas with relatively sparse herbaceous ground cover (McCoy and Mushinsky 1992; Breininger et al.1991). Dense vegetation such as pine plantations create barriers to tortoise movement and can result in isolated patches of habitat while roads can serve as movement corridors (Jones and Dorr 2004; Lohoefener and Lohmneier 1981). However, road beds and ditches may restrict movement somewhat by discouraging road crossings (Gibbs and Shriver 2002) between preferred foraging areas.

Food Habitats/Diet Quality

Tortoises from the two poor habitat treatments (T4 and T5) generally displayed some of the highest selectivity for foraging on the three preferred food items (Fig. 36). With these preferred food items being relatively scarce in their environment, tortoises at T4 and T5 probably spent proportionally more time foraging and moved greater distances in their foraging activities than tortoises from the good habitat treatments. Increased searching and foraging activity by tortoises at T4 and T5 has a bioenergetic consequence in that these individuals probably expended proportionally more energy in activity metabolism than tortoises residing in good habitat areas (Patrick et al. 2006). With increased activity less energy in the form of stored lipids and proteins is available for growth, reproduction, and maintenance of overall health (i.e., such as maintaining immune system competence). The poor quality of the diet of tortoises from T4 and T5 is also reflected in the relatively low levels of essential nutrients and body electrolytes (Fig. 22). Even though the three preferred food items of grasses, forbs, and legumes comprised a greater portion of the diet in tortoises from T6 than from T4 and T5, the diet of individuals from T6 had 20% pine needles which is of very poor quality and would help explain, in part, the relative poor condition, as reflected by several indicators, of tortoises from this treatment (Figs 21, 23, 25, 26).

An important consideration in evaluating the relationship of habitat quality and food quality is the nutritional value of the preferred or ingested food items. Tortoises may be constrained in foraging and plant selection by the ratio of nitrogen to potassium and by certain plants of high N:K (Oftedal and Allen 1996). Garner and Landers (1981) found that legumes are the most important forb in tortoise diets because of their high nutritional value. Effective habitat management for tortoises should include an evaluation of nutritional resources and particular those herbaceous plants that are relatively high in nitrogen content such as legumes.

Population and Landscape Genetics

In general our analyses found that genetic variability among tortoise populations (i.e., including sites and colonies) on Camp Shelby was relatively low. Microsatellite DNA markers have high mutation rates relative to other markers, and are typically highly variable (Goldstein et al. 1999). A benchmark for comparison through a genetic study of tortoise populations can be used from the eastern portion of the geographic distribution using the same microsatellite loci as our study (Table 11). Heterozygosity of tortoises at Camp Shelby was half (51%) that found in eastern populations by Schwartz and Karl (2008). Values for allele richness are only slightly less (93%) for Camp Shelby compared to that of eastern sites, however, this value is misleading because we sampled more than an order of magnitude more individuals than did the other studies. The number of alleles detected in studies is heavily impacted by sample size. This is because low-frequency (i.e., rare) alleles by definition have a greater probability of being detected as sample size increases.

Table 11. Genetic diversity comparison for gopher tortoises at Camp Shelby and in colonies from geographically separated localities in Georgia (n=3) and Florida (n=9) studied by Schwartz and Karl (2008). Data for Camp Shelby are for the entire base, whereas those for Georgia and Florida are averaged across multiple geographic locations throughout each state. Camp Shelby had much lower genetic variation for both allelic richness and heterozygosity.

Location (# localities)	Sample size	Allele richness	Observed heterozygosity
Camp Shelby (1)	309	27.0	0.22
Georgia (3)	17	27.3	0.42
Florida (9)	16	30.6	0.43

Additionally, it is somewhat difficult to interpret genetic variation differences between Camp Shelby and populations from the eastern range because eastern sites are represented by only a single colony with a mean sample size of 16 individuals. If more colonies were sampled within each site at the other reported locations, H_E values may not have changed much but allele richness almost have certainly would have changed. A more realistic comparison for assessing genetic variation would be to use a single site at Camp Shelby colony with the highest genetic variation. Values for this colony (where colony sample size = 20) was $H_E = 0.25$ and allele richness = 18. Using this one colony at Camp Shelby with the highest sample size (n=20),

heterozygosity was slightly higher than the mean value for Camp Shelby (0.25 vs 0.22) and was 59% that of eastern sites (0.25 vs. 0.42). However, allele richness at this single colony at Camp Shelby is only 66% percent that of eastern populations (18 vs. 28.5).

Because the number of tortoises in an area is positively related to habitat quality and population genetic variation is similarly correlated to population size, we predicted that significantly greater genetic variability would be found at sites with higher habitat quality. Although we did not find a significant statistical relationship between habitat quality and genetic variability, we feel that the biological difference and significance is real as illustrated by Figs. 40 & 41. The availability of high quality habitat on Camp Shelby is not limiting, and the number of colonies on poor quality sites is relatively low. Because of this we were only able to sample 5 poor sites of our total 34 sites, and only 4 of these had large enough colony size to include in the analyses of our 25 sites. Having only 4 of 25 sites characterized by poor habitat necessarily resulted in low statistical power to detect any potential relationships between genetic variability and habitat quality. Such a relationship becomes more obvious if statistical comparison is performed on the differences in population size relative to good versus poor habitat sites. Mean population size is 18.1 $\pm$ 2.1 on good and 9.25 $\pm$ 1.8 on poor habitat sites, yet these differences are also statistically non-significant (t_{23} = 1.9, p = 0.08) but most likely biologically significant.

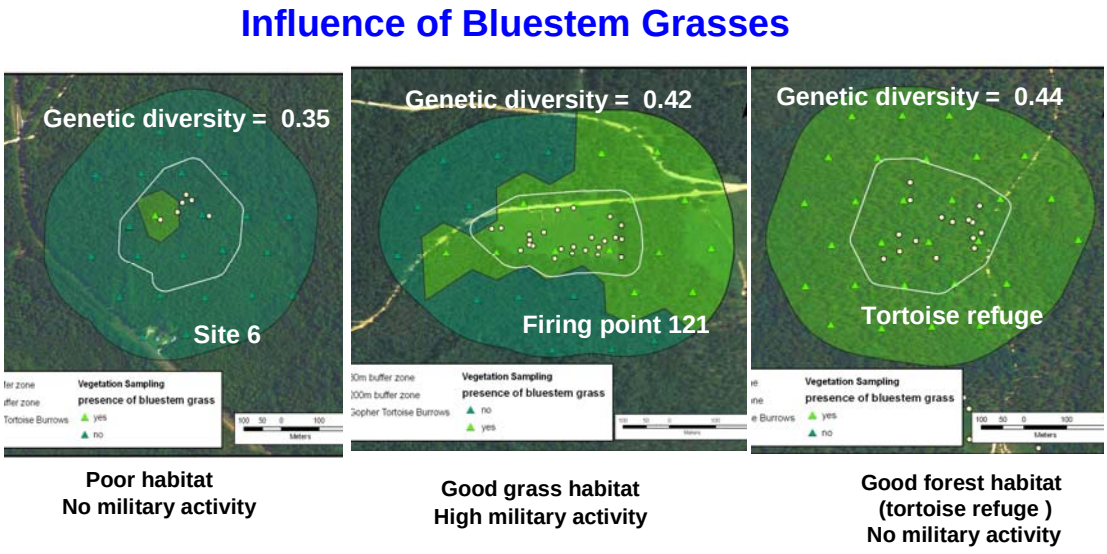
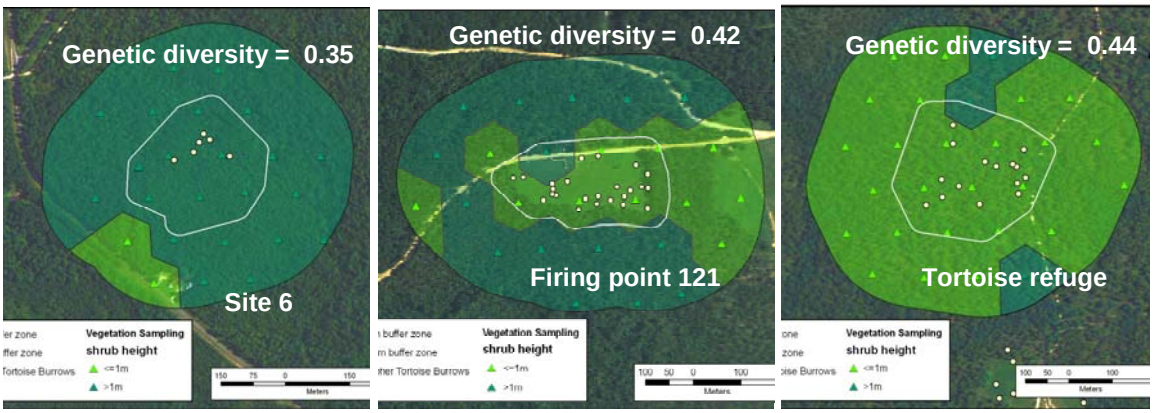


Figure 40. Relationship between habitat quality as demonstrated by the amount of bluestem grasses (light green) and genetic diversity of the gopher tortoises residing at these sites.

Influence of Small and Large Shrubs



Poor habitat
No military activity

Good grass habitat
High military activity

Good forest habitat
(tortoise refuge)
No military activity

Figure 41. Relationship between habitat quality as demonstrated by the amount of small shrubs <l m (light green), large shrubs >1m (dark green) and genetic diversity of the gopher tortoise residing at these sites.

If no biological difference truly exists between genetic variation of tortoises on high and low habitat sites, at least two causal explanations exist. Individual tortoises are found in poor habitats across Camp Shelby, but these burrows tend to be diffusely distributed and not clumped into obvious colonies or populations. A major complication for delineating cause is that it is often the case that tortoises found in poor quality habitat are often located near a colony of individuals on higher quality habitat which together may form a population. This relationship between poor habitat quality and the spatial distribution of colonies is supported by the distribution of tortoise burrows across Camp Shelby where they are clumped in good quality habitat and diffusely spread across adjacent low quality habitats. Additionally, marginalization of tortoises with lower quality/health/genetic variation may not occur at Camp Shelby. Another consideration in evaluating the relationship between genetic variation of tortoises and habitat quality is that the firing points are typically placed on ridge tops which by this "artificial placement" of sites could result in some level of genetic isolation from a habitat perspective.

Genetic structure does not appear to exist for gopher tortoises across Camp Shelby, at least based on F_{ST} values calculated using genetic data of adults from populations across the base. However, a lack of genetic structuring does not necessarily infer a lack of population structuring. Population structure results when populations across a landscape have reduced interactions among them. This situation usually occurs naturally because resources are typically patchy but can also result from human landscape alterations that fragment suitable habitat. Once population structure becomes established, a genetic signature of this structure will eventually follow because of reduced migration among populations, which will in turn reduce gene flow.

Based on the distribution of tortoises on Camp Shelby, population structuring appears to occur across the base due to both natural occurrences of suitable habitat and to anthropogenic habitat fragmentation. Understanding both gopher tortoise life history and the land-use history of Camp Shelby is crucial to interpreting the genetic data and drawing meaningful conclusions. Many of our study sites represent firing points and other land use practice areas that were created within the past 20-40 years (Matt Hinderliter, Camp Shelby, pers. comm.) by converting forest habitat to open-canopy, ruderal habitat, which is preferred by gopher tortoises. Presumably, many of these sites had few to no tortoises prior to habitat conversion and were colonized by tortoises over the years following their creation date. Following this, land between these sites was managed differently, which resulted in varying quality of intervening habitat and suitability for tortoise movement between habitat patches.

Our finding of no base-wide genetic structure is partially explained by the fact that the creation of new habitat followed by colonization of tortoises from nearby populations can result in population admixture, which degenerates (or negates) any preexisting signature of genetic structure. This situation is further explained by the fact that these new habitats were created beginning in 1956 and that gopher tortoises live for > 60 years. Many of the tortoises currently present on our study sites represent the first generation of migrants into these new areas. Therefore, based on what we know about tortoise spatial ecology (i.e., high site fidelity coupled with low dispersal between sites) (Eubanks and Michener 2003), evident population structure, and current patchiness of habitat on Camp Shelby, we predict that genetic structuring will occur over the next generations of tortoises over the landscape of Camp Shelby.

Predicting the degree of future genetic differentiation of tortoises over the spatial scale of Camp Shelby is less straightforward. Although tortoises have high site fidelity and low dispersal between sites, there still may only be weak genetic structuring over time. Biologically, small numbers of effective migrants (i.e., those that actually breed and produce offspring) sustain enough gene flow between populations to maintain genetic similarity (Mills and Allendorf 1996; Wang 2004), even for species with strong philopatry (Alcaide et al. 2009). Even if 95% or more of interpopulation movements of tortoises at Camp Shelby are within home range distances reported for tortoises and only a very small fraction of individuals make periodic long-distance migratory or dispersal treks, this could explain our findings. One effective migrant *per generation* is sufficient to maintain similarity among populations of organisms (Mills and Allendorf 1996; Wang 2004). Given the long generation time of tortoises, this gene flow has fairly high probability of occurrence. In addition, at a few sites, some tortoises have been relocated from other areas which could also help to maintain genetic similarity among some colonies.

SYNTHESIS AND CONCLUSIONS

One of the most complex and challenging issues related to successful TER-S management and sustainability on military lands is that of assessing the effects of multiple environmental factors or stressors which can result from a variety of military and/or non-military causes.

Environmental stressors from military activities such as noise, troop activity, habitat disturbance and fragmentation, and chemical exposure can interact with non-military related stressors such as invasive species and climatic variables (e.g., temperature, drought, fire, floods) to impair the health and fitness of TER-S. Very few, if any, quantitative bioassessment tools are available to environmental managers for assessing the effects of multiple environmental stressors on the health and fitness of wildlife populations. The integrative bioindicator approach applied in this study involves using a suite of biological responses over a range of levels of biological organization to provide a definitive framework for assessing the effects of multiple environmental factors on the health of the gopher tortoise at Camp Shelby. The complexity of ecological systems and the many potential environmental stressors than can impact these systems suggests that no single measurement is adequate to evaluate organism health, and a suite of measures are required to help establish causal relationships between environmental factors and organism health (Kelly and Harwell 1990, Power 1999). Incorporating a variety of response variables (i.e., biomarkers and bioindicators) into the experimental design of environmental assessment studies is necessary in helping to understand causal relationships between environmental factors, organism response, and the biological relevance of such responses. In this study, using the integrated bioindicator approach proved to be a useful management tool for assessing the relationship between the health of a TER-S such as the gopher tortoise and various environmental stressors. Identification of those specific actions and environmental variables (stressors) responsible for injury to TER-S should reduce the uncertainty of environmental management and regulatory decisions resulting in an increased ability to predict the consequences of specific actions or activities on military ranges.

In this study the multivariate bioindicator approach was used to assess the health of tortoises residing in areas characterized by different levels of military activity and habitat quality. Using all the measured bioindicators in a multivariate discriminant analysis procedure revealed that both habitat quality and military activity are important in influencing the health and condition of tortoises at Camp Shelby (Table 12).

Table 12. Diagnostic response profile for tortoises sampled from those combinations of treatments that represent effects of military activity and habitat effects

Main effect	Treatments compared	Effects compared	Diagnostic response profile	Representative bioindicators
Military activity	T1, T2, T3	High, low, & no military activity	Organ dysfunction enzymes, stress hormones, bioenergetic	AST, BUN, cortisol, body condition
Habitat	T1, T5, T6	Good and poor habitat	Electrolyte homeostasis, oxidative stress enzymes, , carbohydrate-protein metabolism	Mg, K, lipid peroxidation, GSH, glucose, protein
Military activity and habitat	T1-T6	Level of military activity and type of habitat	Combination of above variables	Combination of above

A reduced set (7-8) of bioindicators from a total set of about 40 measured variables effectively predicted tortoise health based on differences among the experimental treatments. As functional response groups, the organ dysfunction, carbohydrate-protein metabolism, and stress hormones are the key diagnostic responses that are primarily indicative of habitat effects while bioenergetic, electrolyte homeostasis, and oxidative stress indicators are the principal diagnostic responses that are the key indicators of military activity effects on tortoises. The fact that a number and variety of physiological response groups were important in discriminating among treatments illustrates the importance of using multiple response indicators (or functional response groups) to assess the effects of environmental factors (stressors) on the health of wildlife species. Since habitat quality and military activity both appear important in influencing tortoise health, implications of this finding suggests that a variety of environmental management and mitigation strategies could be implemented to minimize effects of military activities by management and creation of preferred tortoise habitat (discussed in the Management Considerations section).

In assessing the effects of multiple environmental stressors on sensitive wildlife species such as TER-S, application of suites or multiple bioindicators representing different sensitivities, specificities and levels of ecological relevance should also reduce the risk of false positives (Type I error or concluding that effects are occurring when they are not) and false negatives (Type II error or concluding that effects to wildlife are not occurring when they actually are). Use of a single bioindicator or response endpoint, however, may not be adequate to reduce the probability of these types of errors. For example, Beliaeff and Burgeot (2002) reported that using a suite of indicator measures instead of a single biomarker could help avoid false positives and false negatives. Hall and Giddings (2000) also argue for using multiple lines of evidence (measurement of several different response endpoints) for reducing the probability of false positives and negatives. A major goal, therefore, in designing studies to evaluate the effects of multiple environmental factors on the health or fitness of wildlife species of concern is to minimize the probability of false negatives (concluding that effects are not occurring when, in fact, they actually are) for effectively protecting and managing wildlife species of concern on military ranges. For example, at Camp Shelby, environmental monitoring and assessment programs should be adequately designed to minimize the probability of incorrectly concluding that military activity such as habitat disturbance is not affecting the health and fitness of tortoises

when it actually is (Type II error). Making such a false conclusion about the relationship between military activity and fitness of tortoise populations can be minimized by measuring a selective suite of multiple bioindicators that represent a range of responses at different levels of ecological relevance and also at different sensitivities and specificities to environmental factors or stressors.

Both individual level responses and integrative response indicators should be used when assessing effects of environmental factors on the health and fitness of TER-S. Integrative indices such as reproductive integrity and population fitness are primarily structurally-related attributes which are overall indicators of environmental effects on TER-S, but these types of responses, in themselves, provide little information on the underlying mechanisms or causes of observed effects because of their relative insensitivity and slow response times to environmental stressors. Conversely, studies at the organismal and suborganismal (e.g., biomarkers and bioindicators) levels can help provide more functionally or mechanistically-related information on how stressors interact with target biological sites. These more sensitive indicators, however, provide little or no insight relative to the consequences of biologically effects at ecologically-relevant endpoints (Adams 2002). Lower level responses (biomarkers and bioindicators) are crucial for elucidating the mechanistic basis of stress and recovery while studies at higher levels of organization (reproductive integrity, population fitness) are key for understanding the consequences of this stress at ecological relevant levels (Adams 2002). Thus, the importance of organism-level or bioindicator measurements is to provide a pivotal point through which mechanistic understanding and ecological consequences of stress and recovery can be linked (Forbes 1999, Adams et al. 2002, Lee et al. 2008). In the sequence of biological organization from subcellular and cellular levels through populations and communities, the expression of stress at each level has its mechanistic explanation in the levels below and exerts its influence on the levels above (Bartholomew 1964).

The health and fitness of tortoise populations over the landscape of Camp Shelby is primarily related to habitat quality because habitat quality influences tortoise growth rate and therefore body size. Body size and in turn influences reproductive competence (number and quality of eggs) and ultimately population size with population size directly related to genetic fitness and therefore to overall health of tortoises. In this study we found that genetic variation of tortoise colonies or populations was greater at sites with good habitat than at sites with poor habitat and that genetic diversity was also positively related to population size. Based on the distribution of tortoises over the landscape of Camp Shelby, population structuring appears to occur due to both natural distribution of suitable habitat and due to landscape alteration and habitat fragmentation. Many of our study sites are affected by military land-use practices where forest habitat has been converted to open areas with little or no tree canopy. Such open areas are preferred by tortoises because they support a large biomass of ground vegetation such as grasses, forbs, and legumes. To ensure that these sites continue to benefit tortoises both in the long and short term, maintenance of intervening forest habitat is requisite. An indirect benefit of maintaining surrounding forests is that tortoises may migrate into adjacent forest and fewer tortoises would then occupy sites intended for military use thereby minimizing conflict of use on active ranges.

Management Considerations

This study has demonstrated that both habitat quality and level of military activity can influence the health, condition, and fitness of gopher tortoises at Camp Shelby. Training restrictions due to

the presence of TER-S such as the gopher tortoise on military ranges are relatively effective in most cases for minimizing impacts to sensitive wildlife populations, but in situations where risks do occur, various management strategies could be implemented to ameliorate or minimize such risks. Military activities can cause effects on TER-S by both indirect and direct pathways. Direct effects would include such factors as noise, physical damage to burrows and to the animals themselves, and disturbance of preferred nesting and foraging areas. Indirect effects would be manifested primarily through habitat disturbance or destruction, affecting not only the amount of preferred habitat available but also habitat quality.

For mitigating effects of habitat disturbance or fragmentation, prescribed burning is the most proven technique for creating and maintaining preferred gopher tortoise habitat (Landers et al. 1980, Mushinsky and Gibson 1991, Yager et al. 2007). Highest densities of tortoises have been found on lands with mature longleaf pine forests that supports little mid-story vegetation that have been managed with prescribed fire. In addition, reproductive fitness of tortoises is higher in areas that are burned every 3-5 years compared to those unburned for 20^{+} years (Rostal and Jones 2002). Conversely, unburned areas and pine plantations have proven to provide the poorest habitats because of the reduced abundance of herbaceous plants (Hermann et al. 2002, Menges et al. 1993, Varner et al. 2005). A management goal related to prescribed burning would be to provide a range of preferred habitat choices for tortoises by producing a mosaic of vegetation density by altering the frequency and timing of controlled burns (Diemer-Berish 1994). Gopher tortoises benefit from management practices that focus on ecosystem processes and habitat structure. When habitat is managed in a way that results in semi-natural vegetation structure and function, tortoise populations can be self-sustaining (Hermann et al. 2002).

Population stability and site fidelity of tortoises can be enhanced by long-term maintenance of suitable nesting and foraging habitat. Habitat manipulations that reduce canopy cover and increase available (preferred) forage can cause desirable shifts in tortoise population structures over time (Diemer 1992). Habitat management that reduces forest canopy and promotes lush herbaceous ground cover is necessary to maintain health of tortoise populations (Wilson and Mushinsky 1997). Upland habitats with extensive canopy cover can decrease sunlight penetration and hamper the ability of tortoises to attain minimal thermal requirements (thermoregulation) for normal daily activities. Low sunlight can also decrease herbaceous vegetation essential for health tortoise populations (Mushinsky and McCoy 1994). Auffenberg and Iverson (1979) reported that as herbaceous cover decreased tortoise movements and home range increased. The optimum conditions for promoting both growth of adequate herbaceous vegetation for foraging and for thermoregulation of tortoises is to have a canopy that is 20-50% open such as a mature long-leaf pine forest that is regularly maintained with prescribed fire to remove mid-story vegetation (Yager 2005, The Nature Conservancy, personal communication). For optimum management of tortoise habitat, a multi-aged forest is desirable, ranging from treeless areas with high diversity and abundance of grasses and herbaceous plants to areas with tree canopies that cover about 30-50% of the area (Wilson and Mushinsky 1997). Such gopher tortoise habitats should also be managed to maintain existing genetic structure without further isolation of populations (Schwartz and Karl 2006) which could eventually result in lower genetic diversity and reduced population fitness (Hansson and Westerberg 2002).

Many of the habitat alterations on Camp Shelby in which forest habitat was converted to ruderal habitat for military activities appear to have benefitted gopher tortoises based on population sizes at these sites. However, preference for open areas maintained by fire or anthropogenic activity can result in tortoises congregating in relatively high densities (Mushinsky et al. 2003). Therefore, the fitness of tortoises on these sites may be highly dependent upon sufficient availability of resources in the area. If surrounding longleaf pine forest habitat is not maintained by fire, this will result in lower resource availability. Additionally, ineffective management of surrounding forest ecosystems will result in a landscape composed of highly suitable patches of open habitat isolated within a matrix of unsuitable habitat. Such a situation would lead to isolation of tortoise populations and would probably have a negative effect on the longer-term genetic fitness of tortoise populations. To ensure that sites cleared for military activity continue to benefit tortoises both in the short and long term, maintenance of intervening forest habitat is requisite. An indirect benefit of maintaining surrounding forests is that tortoises could migrate into adjacent forest and fewer tortoises would then occupy sites intended for military use therefore minimizing conflict of use on military ranges.

TRANSITION PLAN

The results and recommendations from this project will be provided to interested parties through several modes of distribution. First, guidelines and protocols for experimental sampling design, collection and analysis of samples, and analysis and interpretation of results will be provided to the environmental resource managers at all military installations where gopher and desert tortoises occur. This includes at least 18 installations in the Southeastern US for the gopher tortoise and a few in the southwestern US for desert tortoises. Such dissemination of information has already been initiated at 3 installations in the SE-US including Camp Shelby, Fort Stewart, and Fort Benning. Meetings have been held with environmental resource managers at Camp Shelby and Fort Banning to brief them on the results of this project and to provide recommendations for future studies. In addition, proposals have been submitted through the Dodd ESTCP and Legacy programs to implement research studies on the gopher tortoise at Fort Benning and Fort Stewart that draw on the finding and recommendations of this project. After submitting a preproposal to the Legacy Program, we were encouraged to submit a full proposal which addresses the affects of multiple environmental stressors on gopher tortoises at these installations. An independent proposal, which is based on the findings and recommendations of this SERDP project, will also be submitted to Fort Banning to address some of the specific environmental issues they are having with the gopher tortoise.

Several publications related to the findings and recommendations of this SERDP project are currently being prepared which will also serve as technology and information transfer to environmental resource managers at military installations. These publications include, 1) bimolecular and biochemical responses of tortoises to military activity and habitat disturbance, 2) immune system response to military activity and habitat disturbance, 3) diet quality and food habits of tortoises as a function of military activity and habitat quality, 4) population genetics of tortoises relative to treatment effects and spatial isolation, 5) landscape population genetics which evaluates the effects of various landscape features on the genetic diversity of tortoises, and 6) integration all aspects of this study (i.e., 1-5 above) into one publication to provide a comprehensive overview of the results relative to recommendations and protocols for assessing the effect of various environmental factors on the health and fitness of the gopher tortoise.

The development and application in this study of methodologies and approaches for assessing effects of military activities on TER-S should allow environmental managers at military installations to (1) help manage TER-S under conditions of ongoing military testing and training activities, (2) prioritize the management of environmental stressors or factors according to their relative importance in affecting TER-S fitness and sustainability, and (3) assist in the implementation of adaptive management strategies. The protocols and methodologies developed in this study for the gopher tortoise at Camp Shelby could also be applied to other installations where gopher tortoises occur. In addition, the quantitative methodologies developed for the gopher tortoise should be applicable, with some relative minor modifications to other related species such as the desert tortoise in the southwest. An expected outcome or product of this study is a more targeted and appropriate ecological management strategy for military installations that can be used to (1) help mitigate the environmental stressors that are of the most

concern to TER-S, and (2) provide guidance relative to possible relaxation of those training activities that have minimal effects on the wildlife species of concern.

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APPENDICES

List of Technical Publications

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C.W. Theodorakis, S.M. Adams, C. Smith, J. Rotter, and A. Hay. To be submitted Sept. 2009. Conservation toxicology: effects of military activity and habitat quality on DNA damage and oxidative stress in gopher tortoises on an army training facility. Environ. Toxicol. Chem.

S.C. Richter, J.A. Jackson, S.M. Adams, M.G.Hinderliter, D. Epperson, and C.W.Theodorakis. To be submitted July-Aug. 2009. Conservation genetics of federally threatened Gopher Tortoises (*Gopherus polyphemus*) in the western portion of their range. Conservation Genetics.

M.G. Hinderliter, S.M. Adams, and D. Epperson. To be submitted fall 2009. Reproductive dynamics of the gopher tortoise at Camp Shelby, MS. relative to military activity and habitat quality. J. Widlife Management.

S.M. Adams, M.J. Peterson, A.D. Hewitt, T.F. Jenkins, M.G. Hinderliter, and L.Y. Yager. 2005. Assessing the health and fitness of TES on military ranges: Importance of incorporating integrated chemical and habitat characterization studies in experimental designs. Abstract for 2005 annual SERDP meeting.

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